The Role of Homologous Recombination Repair in the processing of G2-chromosomal breaks and maintenance of G2-checkpoint

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Aashish Soni

aus

Sagar, Madhya Pradesh, Indien

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1. Gutachter: Prof. Dr. George Iliakis

2. Gutachter: Prof. Dr. Ann Ehrenhofer Murray

Vorsitzender des Prüfungsausschusses: Dr. Juergen Thomale

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“An ounce of practice is better than tons of theory”

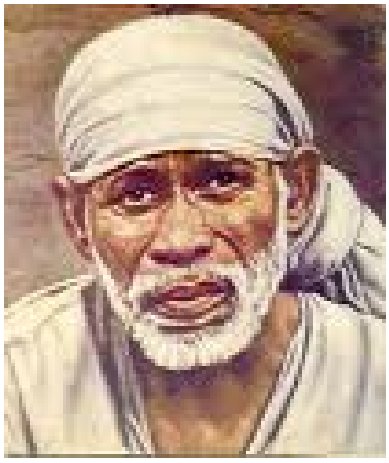
(Swami Sivananda Saraswati)

“The thing always happens that you really believe in; and the belief in thing makes it happen”

(Frank Lloyd Wright)

Dedicated to

My beloved Shirdi Sai baba



॥श्री सद्रगुरु साईनाथार्पणमस्तु । शुभं भवतु ।।

शिरडीस ज्याचे लागतील पाय, टळती अपाय सर्व त्याचे

माझ्या समाधीची पायरी चढेल, दुःख हे हरेल सर्व त्याचे

जरी हे शरीर गेलो मी टाकून, तरी मी धावेन भक्तासाठी

नवसास माझी पावेल समाधी, धरा द्रढ बुद्धी माझ्या ठायी

नित्य मी जिवंत जाणा हेंची सत्य, नित्य घ्या प्रचीत अनुभवे

शरण मज आला आणि वाया गेला, दाखवा दाखवा ऐसा कोणी

जो जो मज भजे जैशा जैशा भावे, तैसा तैसा पावे मीही त्यासी

तुमचा मी भार वाहीन सर्वथा, नव्हे हें अन्यथा वचन माझे

जाणा येथे आहे सहाय्य सर्वांस, मागे जे जे त्यास ते ते लाभे

माझा जो जाहला काया वाचा मनीं, तयाचा मी ऋणी सर्वकाळ

साई म्हणे तोचि, तोचि झाला धन्य, झाला जो अनन्य माझ्या पायी

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°C	degree Celsius
%	percent
~	Approximately
γ-H2AX	Phosphoryaltion of Histone H2AX at Ser-139
53BP1	p53 binding protein
AT	ataxia–telangiectasia
ATLD	ataxia–telangiectasia-like-disorder
ATM	ataxia-telangiectasia-mutated
ATR	ataxia-telangiectasia and rad3 related
ATRIP	ATR-interacting protein
B-NHEJ	backup pathway of non-homologous end-joining
BRCA1	breast cancer susceptibility gene 1
BRCA2	breast cancer susceptibility gene 2
BRCT	breast cancer C-terminal
Cdk	cyclin-dependent protein kinase
cm	centimeter
Chk1	Checkpoint kinase 1
Chk2	Checkpoint kinase 2
CtIP	C-terminal binding protein interacting protein
CSR	class switch recombination
Cys	cysteine
DDR	DNA damage response
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DNA-PK	DNA dependent protein kinase
DNA-PKcs	catalytic subunit of the protein DNA-PK
D-NHEJ	DNA-PK-dependent non-homologous end-joining
ds	double stranded
DSB	DNA double strand break
e.g.	exempli gratia
eV	electron Volt
FA	Fanconi anemia

FACS	fluorescence activated cell sorting
FBS	fetal bovine serum
FITC	Fluorescein isothiocyanate
G1	Cell cycle phase Gap 1
G2	Cell cycle phase Gap 2
GFP	green fluorescent protein
Gy	Gray
H3ps10	Histone H3 phosphorylated at serine 10
HDAC	Histone deacetylase
h	hour
His	histidine
Hj	Holiday junction
HRR	homologous recombination repair
hSSB1	ssDNA-binding proteins
HST	Histogram
IR	ionizing radiation
k	kilo
kDa	kilo Dalton
KU-55933	KUDOS55933 (2-morpholin-4-yl-6-thianthren -1-yl-pyran-4-one)
keV	kilo electron Volt
LET	linear energy transfer
Leu	leucine
Lig4	Ligase4
LMDS	locally multiplied damage sites
MEF	mouse embryo fibroblast
min	minute
MI	mitotic index
mm	millimeter
M-phase	cell cycle phase Mitosis
MPF	mitosis promoting factor
MMEJ	microhomology-mediated end-joining

MRN complex	Mre11-Rad50-Nbs1 complex
µm	micro meter
ng	nano gram
nm	nano meter
NBS	Nijmegen Breakage Syndrome
PBS	phosphate-buffered saline
PCC	premature chromosome condensation
PFGE	pulsed field gel electrophoresis
PI	propidium iodide
PIKK	phosphoinositide-3-lipid family of kinases
PP2A protein	phosphatase 2
RPA	replication protein factor A
RNase	ribonuclease
rPAb	rabbit polyclonal antibody
rpm	rounds per minute
Ser	serine
s	second
SCID	severe combined immunodeficiency
S-phase	cell cycle Synthesis phase
ss	single stranded
SSB	single strand breaks
Thr	threonine
Tyr	tyrosine
UV	ultraviolet light
wt	wild type
XRCC	X-ray cross complementation group

1. Abstract

DNA double strand breaks are potent inducers of cell cycle checkpoints, DNA damage response (DDR) signalling and repair pathways. Ionizing radiation (IR) efficiently induces DSBs. G2-M checkpoint activation delays/stops cell division to facilitate repair of these lethal lesions. According to the classical view proteins of PI3K kinase family ATM and ATR have been shown to be the key players in the activation of this checkpoint. In higher eukaryotes, two main repair pathways named NHEJ and HRR are responsible for the repair of DSBs.

For many years, HRR has been considered to be a minor pathway of DSB repair in higher eukaryotes. This opinion is based on the fact that none of the cell lines deficient in important HRR genes displays obvious DSB repair defects although they are clearly radiosensitive to killing. It is therefore thought that in higher eukaryotes IR induced DSB repair is practically exclusively undertaken by the D-NHEJ pathway; on the other hand the role of HRR is only evident on the repair of I-SceI induced site directed DSBs in integrated genomic loci. Thus, how HRR contributes to the repair of IR induced DSBs remains unknown.

In the work presented here, we study the repair of IR induced DSBs through their transformation into G2-chromosomal breaks. Our results show the direct involvement of HRR in the repair of a subset of IR induced DSBs which are associated with the formation of G2-chromosomal breaks. The D-NHEJ repair pathway does not seem to play a role in the repair of these breaks.

Our results also demonstrate that HRR is a key player in the activation and maintenance of the G2-checkpoint. We employed flow cytometry based determination of mitotic index after exposure to 1Gy X-rays using phosphorylated histone-H3 as a marker. Chinese hamster cells deficient in RAD51 paralogs RAD51B, RAD51C, RAD51D, XRCC2 and XRCC3 show a remarkable defect in G2-checkpoint activation, while cells deficient in NHEJ components KU80, DNA-PKcs and XRCC4 show strong G2- checkpoint activation when compared to the wild type cells.

This is, to our knowledge, the first report implicating HRR in the repair of a subset of DSBs with the potential of forming chromosome breaks, as well as in the development and maintenance of DNA damage induced cell cycle checkpoints.

Key words: Ionizing radiation, DNA-double strand break, D-NHEJ, HRR, G2-checkpoint, Chromatid breaks

2. Introduction

2.1 Ionizing radiation and induction of double strand breaks

Ionizing radiation (IR) consists of subatomic particles and electromagnetic waves which carry enough energy to kick out electrons from atoms or molecules. X-rays and gamma rays are electromagnetic waves, whereas neutrons, electrons and protons are particulate. Particulate radiations tend to be more densely ionizing. The energy deposition in tissues and cell culture systems varies considerably depending not only on the type of radiation but also on the energy of the radiation (1). Linear energy transfer (LET) is a measure of the energy transferred to per unit distance of material as an ionizing particle travels through it. The particles which are slow moving i.e. alpha particles have higher LET than X-rays or gamma-rays. It is clear that between the initial physical processes and the eventual biological outcome, chemical reactions of free radicals and ion radicals are involved (2). At the molecular level, energy deposition by IR is non homogenous. When cells are exposed to ionizing radiation, radiochemical damage can occur either by direct action or indirect action. Direct action occurs on the DNA when alpha particles, beta particles or x-rays create ions on it, which physically break the sugar phosphate backbone or damage bases (Figure 2.1a, page 4). The indirect action occurs through generation of hydroxyl radicals by radiation in the surrounding water, which then damages the DNA (Figure 2.1b, page 4). The indirect effect is responsible for the majority of damage causing the more severe biological effect of IR (3). Radiation is measured in the SI units of Gray (Gy) describing the amount of energy absorbed by a unit of mass. The unit of 1 Gy equals one Joule of energy absorbed by one kg of mass (1J/kg).

Direct Action

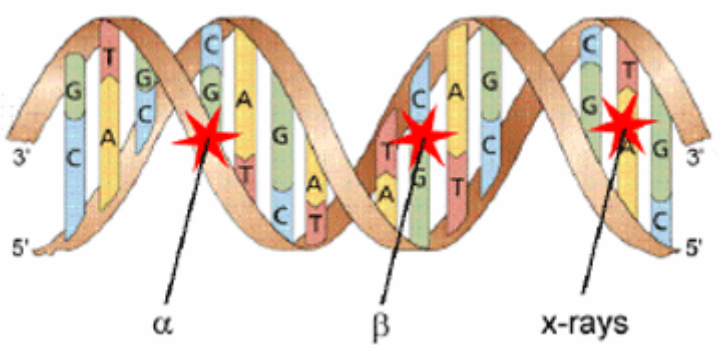


Figure 2.1a

Indirect Action

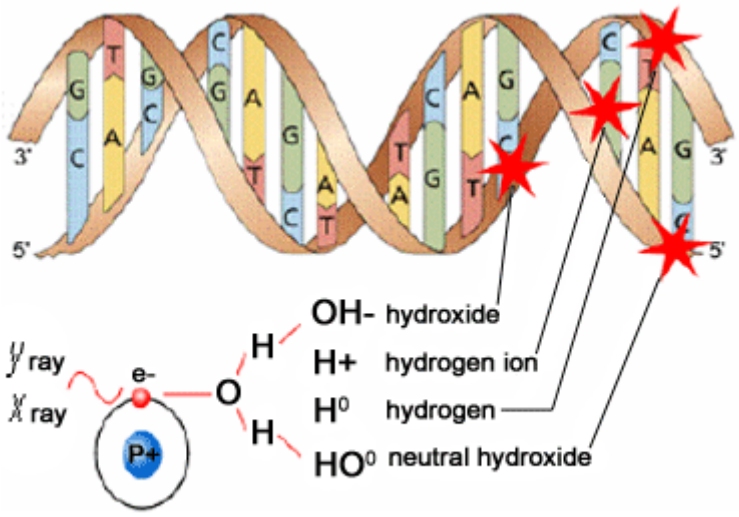


Figure 2.1b

(Pictures opted from www.cna.ca/curriculum/cna_bio_effects_rad/dir...)

Both particulate and electromagnetic radiations can cause direct damage. However, heavily charged particles, such as alpha particles, have a greater probability of causing direct damage compared to X-rays, which cause more damage by indirect effects. The chemically reactive species produced by IR in the water react within 2-3 nm from their site of origin with DNA and when they occur in clusters at the ends of

electron tracks, they generate locally multiply damaged sites (LMDS). LMDS comprise diverse forms of damage including single strand breaks (SSBs) and base damages that can combine to form DSBs (4). In this way, exposure to IR generates a spectrum of DNA damages, among which the most genotoxic lesions are DSBs. With increasing LET, LMDS are produced with higher probability. This does not imply that the absolute DSB yield would increase, because the energy deposited per LMDS also increases relative to low LET damage.

2.2 Consequences of DSBs in eukaryotes

IR does not cause instantaneous cell demise, but compromises instead further cell proliferation by generating DNA damage causing chromosomal aberrations and by inducing genomic instability. The genomic instability is one of the most important causes of carcinogenesis (5,6). DSBs generate chromosome fragments, which can miss-rejoin and generate problems in genome organization and in DNA sequence. These processes can cause shuffling of important genes. DSBs differ from other types of DNA lesions, in the sense that both strands of double helix are damaged, which prevents the use of the complementary DNA strand as a template for repair. As a result, DSB repair has to use alternative mechanisms to maintain the sequence of the DNA, and when these mechanisms fail chromosomal aberrations are generated. Common chromosomal aberrations include the loss or gain of whole chromosomes or chromosome fragments. Loss of large regions of a chromosome can inactivate tumour suppressor genes, while amplification of chromosomal regions may promote tumorigenesis by the activation of proto-oncogenes (7). When chromosome arms are exchanged, the aberration formed is known as a translocation, which can be either balanced or unbalanced and be associated with the deregulation of gene expression at the generated junctions (8).

2.2.1 DSBs and chromosomal aberrations

It is widely accepted that DSBs are involved in the formation of chromosome breaks. Thus, is has been reported that DSBs induced by restriction endonucleases cause chromosomal aberrations (9,10).

There are two prevalent theories of chromosome aberration formation. The first theory is known as breakage and reunion (B&R) theory proposed by Lea and Sax in

1946. According to this theory a primary break is a complete severance of the chromatid "backbone" or "Chromonema", leaving open break-ends capable of restituting (reforming the original sequence), illegitimate rejoining (fusing pair wise with foreign ends to form structural exchanges), or remaining open to appear at metaphase as "breaks" (terminal deletions). It is implicit in the B&R theory that "breaks" visible at metaphase are the residues of primary breaks that have neither restituted nor rejoined, i.e. we are viewing the potential progenitors of exchange aberrations (11). The second theory is known as the exchange theory proposed by Revell (1959 and 1963), according to which, all visible breaks are discontinuities, and therefore these visible breaks are the result of structurally incomplete intra-arm intrachanges, from those that are simply the result of failed restitution or rejoining of primary breaks (12).

It is known to be a consistent and general phenomenon observed for normal cells with intact DNA repair mechanisms that cells exposed to IR in G1 or G0 arrive in their first mitosis with aberrations are virtually all of the chromosome-type, while after irradiation in S or G2, aberration are virtually all of the chromatid-type. While some cells irradiated in early S-phase may occasionally contain both chromosome-type and chromatid-type aberrations in the same cell, mostly chromatid-types are seen for irradiation at this time. It is also of considerable interest that the rule of "chromosome-type aberrations" after G1 or G0 irradiation, and of "chromatid-type" after S or G2 irradiation applies only for IR or agents known to produce prompt DSBs, such as neocarzinostatin, Bleomycins etc (13).

In contrast to IR, DNA damaging agents that do not produce prompt DSBs also do not produce chromosome-type aberrations in the first mitosis after treatment in G1 or G0, but instead, produce chromatid-type aberrations (14). Chromatid-type aberrations can of course become derived chromosome-type if the cells bearing them survive into the second or later generations. The cell's survival, when it bears a chromosome-type aberration will largely depend on whether the aberration is a symmetrical inter-change, i.e. an inversion or balanced translocation, which is not lethal, or its asymmetrical counterpart, i.e. an acentric or a centric ring or a dicentric with its acentric fragment, which is generally lethal. The latter form of chromosome aberrations are nearly always lethal for a normal diploid cell because of the large genetic loss associated with the acentric fragment loss in the cell progeny (15,16).

Thus, whether the aberration that is induced appears as a chromosome or chromatid type in the first mitosis, cells that carry them will generally die. Cells that survive with aberrations that are not lethal have also the potential to produce genomic changes of the type seen in malignancies. Chromatid exchanges occur at a much lower frequency than breaks. Exchanges represent the miss-rejoining of at least two DSBs (17).

2.2.2 DSBs can also be necessary intermediates in important processes

DSBs can actually be beneficial for the cell when they occur in a controlled manner in the context of specialized events that require rearrangements in the genome. Controlled enzymatic induction of DSBs is observed during the development of the immune system and the generation of genetic diversity during meiosis (18-20). DSB formation plays a very important role during meiotic homologous recombination in lower as well as in higher eukaryotes (21). It is safe to assume that cellular DSB repair mechanisms maintain continuous activity to ensure that the occurrence and resealing of DSBs will leave the cell unharmed. Also, when DSBs are inflicted into the genome by damaging agents, such as IR or radiomimetic drugs, the threat to cell life is sufficiently serious to set in motion, within minutes, decisive DNA-damage response pathways.

2.3 Mechanisms of DSB repair

In higher eukaryotes IR induced DSB can in principle be repaired by homologous recombination repair (HRR) and non-homologous end-joining (NHEJ). These repair pathways are supported by a well regulated DNA damage response (DDR) signalling cascade that culminates with the activation of cell cycle checkpoints. These two DSB repair modes differ in their requirement for a homologous DNA template and in the fidelity of DSB repair. HRR ensures accurate DSB repair, while D-NHEJ is considered as error prone pathway. The relative contribution of these two DSB-repair pathways is likely to differ depending on the stage of the cell cycle (22). HRR is thought to be more efficient in the S and G2 phases of the cell cycle due to the availability of sister chromatids (23). Theoretically, HRR could occur in G1 as well, by using the homologous chromosomes as a template for repair, but such events are hampered by the highly-ordered chromatin structure and the clearly defined and well

separated in the nucleus of the territories of the homologous chromosomes, which makes such events very rare. The preferred use of a sister chromatid as a repair template probably reflects a proximity effect.
In the absence of a sister chromatid, DSB repair in G1 phase could still efficiently occur through D-NHEJ. Micro-homology dependent alternative pathways are also reported, which are functional in the absence of D-NHEJ (24,25). It is very important that cells accurately repair DSBs, because failure to do so can lead to mutations or genome rearrangements, and indeed a single un-repaired DSB is sufficient to kill a cell (26,27). The following sections describe DDR signalling, checkpoint activation and the pathways of DSB repair in detail.

2.3.1 DDR signalling and cell cycle checkpoints

The recognition and repair of DSBs is a complex process that draws upon a multitude of proteins. This is not surprising since this is a lethal lesion if left un-repaired, and a lesion that can contribute to genomic instability and the consequential risk of cancer and other pathologies. DSBs activate DDR signaling, which results in the activation of cell cycle checkpoint pathways that facilitate specific DNA-repair mechanisms in the different phases of the cell cycle. Checkpoint-arrested cells resume cell-cycle progression once damage has been repaired, whereas cells with un-repairable DNA lesions undergo checkpoint adaptation, permanent cell-cycle arrest, or apoptosis. DDR signaling collectively involves sensing of DNA damage, signal its presence and mediate the cell cycle responses. These processes are regulated by post–translational modifications such as phosphorylation, ubiquitinylation and acetylation.

2.3.1.1 DNA damage sensing

IR induced DSBs act as a signal, which is sensed by DDR signalling proteins. Replication protein A (RPA) and the MRN complex are hypothesized to act as sensors of DNA damage and to help activate the DNA-damage-response-transducing kinases, e.g. ATM and ATR (28,29). RPA is the major ssDNA-binding protein and is essential for DNA replication, repair and recombination (30). MRE11 protein binds both ssDNA (single stranded DNA) and dsDNA (double stranded DNA) in a sequence independent manner. The nuclease activity of MRE11 processes the

3'-ssDNA, a binding site for RPA (31). The RPA–ssDNA complex inhibits any further nuclease activity and initiates the recruitment of the repair machinery (32,33). MRE11 is an 81-kDa protein that has an amino-terminal nuclease domain and two DNA-binding motifs. The mutated MRE11 gene is responsible for Ataxia-telangiectasia-like disorder (ATLD). The clinical features of ATLD are similar to those in patients with AT, with a progressive, but slower and later onset of cerebellar ataxia (34).

2.3.1.2 DNA damage signalling

ATM plays a central role in DSB signalling. ATM normally exists as an inactive dimer, which dissociates into active monomers after sensing DSBs induced by IR. During this process, ATM undergoes intermolecular phosphorylation on Ser1981, Ser367, and Ser1893 (35). ATM autophosphorylation at Ser1981 was shown to be necessary for both monomerization and binding to regions flanking DSBs (36). In addition to the phosphorylation process, dephosphorylation of ATM by several phosphatases i.e. PP2A is also involved in the regulation of ATM activity (37). In the presence of DSBs, ATM phosphorylates a variety of protein targets, and activates several signalling cascades. ATM induces cell cycle arrest at the G1/S, intra-S, and G2/M phases through the action of intermediates, like p53 and Chk2 (38). The active ATM phosphorylates the histone H2A variant H2AX at serine 139 (known as γH2AX), a critical substrate of the DNA signalling machinery, in response to DSBs (39,40). H2AX phosphorylation occurs at megabase regions surrounding the DSBs within seconds of DNA damage, suggesting that H2AX phosphorylation may be a critical component in early DNA damage signal transduction (41). The activated ATM kinase phosphorylates a set of other substrates, e.g. NBS1, also known as nibrin, on serine 34 (42), MRE11 (43), MDC1 at Ser-635 and Ser-645 (mediator of DNA damage checkpoint 1) (44), BRCA1 (45), SMC1 (structural maintenance of chromosome 1) on serines 957 and 966 (46), CHK2 [checkpoint kinase 2] (47), p53 on Ser-15 (48), RPA (49), FANCD2 (Fanconi anemia complementation group D2 isoform) on Ser-222 (50), Artemis (51), and DNA-PKcs at Threonine-2609 (52).

Phosphorylated H2AX forms foci at and near the sites of DSBs, colocalizing with ATM, MDC1, 53BP1, BRCA1, the MRN complex, and many other DNA damage signalling and repair proteins (53,54). The initial migration of factors to DSBs does

not require γH2AX, but the subsequent association with chromatin regions distal to the break site is dependent on the phosphorylation marks on γH2AX (55). Therefore, γH2AX is suggested not to recruit but to retain proteins, including the MRN complex, 53BP1, and BRCA1 at the sites of DNA damage (54).

ATR, which is classified as a member of the same PIKK family as *ATM*, is also an important DDR signalling protein. It is involved in the initiation of HRR via Chk1, a substrate kinase of ATR. Chk1 facilitates HRR through RAD51 phosphorylation, which attenuates the interaction between RAD51 and BRCA2. The regulation of this interaction is required for the appropriate chromatin binding of RAD51 via Chk1 and Chk2 (56). This indicates the possibility of involvement of ATM in HRR regulation through the activation of the ATR-Chk1 pathway. The proteomic analysis of the components of DNA repair machinery revealed an extensive network of more than 700 proteins; all of which are phosphorylated by ATM and ATR in response to DNA damage (57). One of the targets of ATM and ATR kinases is the MRN complex (MRE11/RAD50/NBS1), a highly conserved protein complex involved in DNA replication, DNA repair, telomere maintenance, and signalling to the cell cycle checkpoints (58). The MRN complex plays a critical role in the DNA damage sensing, signalling and repair mechanisms, as well as in the maintenance of chromosomal integrity. According to the classical view, ATM is activated by IR, while ATR is activated by UV light. ATR and ATM are therefore considered to be activated by separate signals and are specialized in their responses to DNA damage (59). Recently it is reported that ATM substrates involved in cell cycle checkpoint signalling can be phosphorylated independently by ATR (60).

While *ATM* knockout cells are viable, *ATR*$^{-/-}$ and *CHK1*$^{-/-}$ both are lethal in mammalian cells, indicating that the ATR/Chk1 pathway plays an essential and ATM-independent role in mammalian cells. The function of ATM has been extensively studied in cell lines derived from ataxia telangiectasia patients that lack expression of the ATM protein. The lack of comparable cell lines for ATR has impaired analysis of its specific activities. Overexpression of catalytically inactive versions of ATR indicates that it is required for checkpoint responses after treatment of cells with agents that cause various forms of DNA damage or block replication (61-64).

2.3.1.3 Cell cycle checkpoints

The outcome of damage induced DDR signalling is the activation of cell cycle checkpoints. The division of a cell is under the control of cell-cycle checkpoints in the G1, S, G2, and M phases, the activation of which leads to arrest of the normal division process (26). It is generally assumed that cell-cycle arrest is necessary to provide the cell with time to effectively repair the DNA lesion. In the case of DSBs, cell-cycle arrest is thought to be primarily mediated by members of the phosphatidylinositol 3-kinase-like kinase (PI3KK) family, which are activated upon the onset of DNA breakage (26,59,65). In human cells, the activation of at least two PIKK signalling kinases- the ataxia telangiectasia mutated (ATM) and ataxia telangiectasia related (ATR) proteins – leads to phosphorylation and activation of the central cell-cycle regulators p53, Chk1 and Chk2, which in turn facilitate G1 and G2 arrest via down regulation of Cdk2/Cyclin E and Cdk2/Cyclin B1, respectively (26). When the cell division is halted through checkpoint activation, several sets of enzymes come into play to repair the damage.

The activation of the G2 checkpoint delays mitotic entry in the presence of DNA damage. Because the spindle checkpoint at mitosis only responds to spindle damages or miss-attached chromosomes (66), the G2 checkpoint is the last chance for correcting genomic damage prior to cell division (67). This fact probably explains its importance in the overall checkpoint response. Proteins participating in the activation of the G2 checkpoint are highly conserved in eukaryotes (68). It is well documented that the ultimate target of the initiated G2 checkpoint signal transduction pathway is Cdc2 (Cdk1) (69,70). In G2, this cyclin dependent kinase (Cdk) is activated after binding to cyclin B1, by a phosphorylation at Threonine-161 and a dephosphorylation at Threonine-14/Tyrosine-15 (69). In this way Cdk1 is associated with cyclin B1 and forms the so called mitosis promotion factor (MPF). See Figure 2.2 (page 12) for the schematic representation of the G2-checkpoint activation.

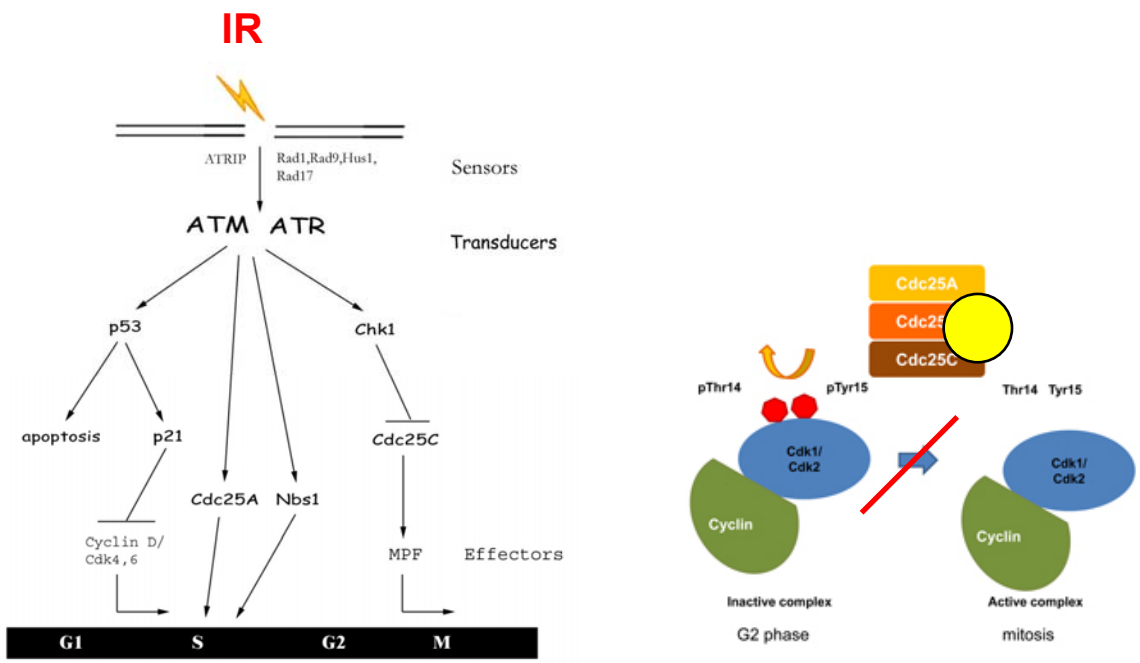


Figure 2.2: Role of ATM and ATR in checkpoint activation (left). Activation of G2-checkpoint through phosphorylation of Cdc25c. It is shown that phosphorylated Cdc25c can not dephosphorylate the cyclinB1/Cdk1 complex. Thus entry into mitosis is inhibited (right).

(Modified from http://www.ruf.rice.edu/~rur/issue1_files/renthal.html & http://www.dkfz.de/en/f045/index1.html)

It has been shown that cells lacking ATM have a defective IR-induced G2 checkpoint (71-75). However, Ford et *al.* (1984) argued that this AT phenotype was an artefact of the selective elimination of a sub population of AT cells (i.e. those die in G2). Further reports (76,77) support the finding of an increased G2 block in AT using different AT cell types. Beamish and Lavin (1994) resolved these apparently contradictory responses by considering the stage of the cycle at which the AT cells are irradiated: irradiation in G1 and especially S phase results in significant delay in G2, while irradiation in G2 gives reduced delay into mitosis and the following S phase (78). It was also shown that abrogated G2-checkpoint in AT cells compromises the repair of chromosomal breaks (74).

ATR is shown to be recruited to the sites of ss-DNA bound by RPA. This recruitment occurs by a mechanism that requires ATRIP (79,80). Activated ATR mediates phosphorylation of protein kinase Chk1, which promotes the activation of the G2-checkpoint and the HRR pathway of DSB repair (81,82). ATR is essential for

embryonic development (83,84), but expression of doxycycline inducible kinase dead dominant-negative ATR prevents G2-checkpoint responses after exposure to UV and ionizing radiation (61).

Caffeine, a non-specific inhibitor of ATM and ATR sensitizes AT cells to IR-induced killing, suggesting that the target of caffeine in AT cells might be ATR (85). In this paper, it is shown for the first time that the prolonged G2-accumulation in irradiated AT cells is regulated by the *ATR/CHK1* pathway. The IR induced, prolonged G2 accumulation in AT cells is abolished by blocking the *ATR-CHK1* pathway, indicating that the over-activated *ATR-CHK1* pathway is responsible for the prolonged G2 accumulation in irradiated AT cells.

The breast cancer associated gene BRCA1 is also shown to be needed for G2-DNA damage checkpoint control, as well for HRR mediated repair of DSB (86,87).

2.3.2 Homologous recombination repair

Rad51 dependent HRR is usually an error-free repair pathway dependent on homologous sister chromatid strand availability as a template, which restricts its activity to S and G2 phase of the cell cycle (23). Using an intact DNA molecule as template allows the accurate repair of DSBs. The basic HRR machinery and its regulation are remarkably conserved among eukaryotes (88). In higher eukaryotes, HRR is driven by the proteins of the Rad52 epistasis group, which includes Rad51, Rad52, the Rad51 paralogs; Xrcc2, Xrcc3, Rad51B, Rad51C, Rad51D, and Rad54/Rad54B (89). In human cells, the products of breast cancer susceptibility genes, BRCA1 and BRCA2 (FANCD1), are also involved in HRR (90).

The process of HRR is relatively slow but provides accurate repair of damaged DNA regions. It involves the following distinct steps (1) processing of DNA ends, (2) search for homology, strand invasion and formation of Holiday junction, (3) DNA synthesis, branch migration, and (4) final resolution of Holiday junction intermediates (see Figure 2.3, page 16). The initial cellular response to DSBs is mediated by ATM and one of the subunits of MRN complex Nbs1. The MRN complex is involved in primary DNA-end recognition and nucleolytic processing of the broken DNA ends into 3'-end single-stranded DNA tails. CtIP [CTBP (C-terminus-binding protein of adenovirus E1A)-Interacting protein] is ubiquitinilated by BRCA1, thus is recruited to

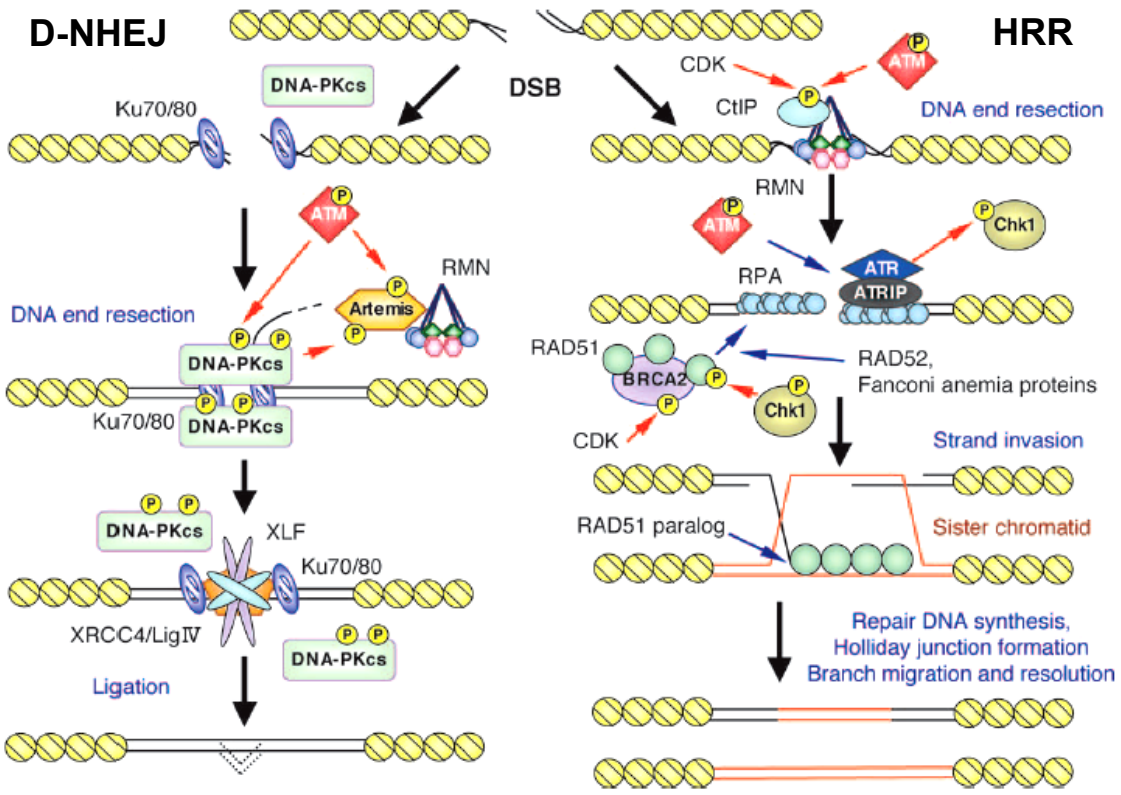
DSBs together with MRE11, promotes DSB ends resection and results in the formation of recombinogenic 3'-overhangs (91,92). CtIP interacts with the MRN complex and enhances MRE11's ssDNA dependent endonuclease activity. Although Mre11 and CtIP promote DNA end resection in the initial step of HRR, these two enzymes do not exhibit processive nuclease activity, which suggests for the existence of at least one more nuclease involved in this step. Bolderson *et al.* have shown the critical requirement of Exonuclease1 (Exo1) for resection of DSBs repaired via HRR (93). The resulted 3'-overhangs, formed after the end resection step are rapidly bound by the ssDNA-binding protein RPA (replication protein A), which in general prevents the formation of secondary structures in the DNA, thus facilitating the formation of Rad51 nucleoprotein filament. Once resection is progressing, the ssDNA serve as a substrate for HRR but also leads to ATR activation that might also act to promote HRR. Consistent with this idea, ChK1 has been shown to facilitate HRR, at least in part by mediating phosphorylation of the key HRR protein, RAD51 (82). The DNA strand invasion and homology search steps of HRR require formation of a Rad51 nucleoprotein filament composed of thousands of Rad51 monomers bound to ssDNA. The formation of Rad51 nucleoprotein is also facilitated by the Rad51 paralogs; Rad51B, Rad51C, Rad51D, Xrcc2 and Xrcc3. Since RPA binds more avidly to ssDNA than Rad51, additional activities are required to load Rad51 on to RPA-coated ssDNA and to displace RPA. In mammalian cells, a critical mediator complex appears to include BRCA1/BARD1 and BRCA2 (FANCD1), probably bridged by the FANCN/PALB2 (partner and localizer of BRCA2) (94,95). BRCA2 is involved in the direct loading of Rad51 (96). The Rad51 nucleoprotein filament then captures duplex DNA and searches for homology. Studies using bacterial RecA indicate that the homology search probably occurs by way of random collisions between the nucleoprotein filament and the duplex DNA, thereby testing segments of the dsDNA in an interactive fashion until homology is found. Following synapsis, the invading strand sets up a D-loop intermediate, whereby the 3'-end primes DNA synthesis using the duplex DNA as a template. It is presently unclear which polymerase (s) mediate D-loop extension in vivo, but Pol η (DNA polymerase η) can perform this function in vitro (97). If the D-loop captures the second end of the break, the HJs (Holliday junctions) are formed which could yield upon appropriate resolution crossover or non-crossover products. However crossing over is rare during somatic HRR (23,98,99). The later steps of the process include polymerization of

nucleotides to restore degraded DNA strand and resolution of the recombination intermediates. The heterodimeric Mus81-Eme1 structure-specific endonuclease plays a role in the cleavage of D-loops, 3' flap structures, nicked HJs and aberrant replication forks, suggesting its roles both upstream and downstream of HRR (100). Nagaraju and co-workers has shown that Rad51 paralogs Xrcc2 and Xrcc3 control the termination of gene conversion between sister chromatids (101).

The Rad51 paralogs Rad51B, Xrcc2 and Xrcc3 are involved in HRR and the maintenance of chromosomal stability. DT40 cells deficient in *RAD51* paralogs showed reduced levels of HR and a high percentage of cells have chromosomal aberrations (102). Murine cells deficient in *XRCC2* or *XRCC3* gene show low levels of HRR (23,103) and high levels of chromosomal aberrations and/or chromosomal miss-segregation at mitosis (104,105). It is important to consider that null mutations in core HRR genes such as *RAD51*, *BRCA1* and *BRCA2* are lethal in dividing cells, whereas null mutations in the Rad51 paralogs are embryonic lethal but not cell lethal (106).

Several DDR signalling proteins are implicated in the regulation of HRR after induction of DSBs. I-SceI induced HRR was shown to be reduced several-fold in $H2AX^{-/-}$ cells (107). ATR has shown to affect HRR but not NHEJ (108). While ATM has been shown to promote HRR following IR induced DSBs (109), ATM deficiency did not reduce the HRR repair frequency of an I-SceI induced DSB (110). MRN generally promotes HRR rather than direct rejoining repair (NHEJ), and this may suggest how MRN could suppress tumorigenesis through the prevention of mutations in genomic DNA. Although ATM phosphorylates many factors involved in the HRR pathway such as BRCA1, Nbs1 and H2AX (57), the role of ATM in HRR regulation is still unclear (111).

Adimoolam et *al.* (2007) showed that PCI-24781, an HDAC inhibitor inhibits HRR via down-regulation of Rad51 in Chinese hamster and human cells (112).



[From IJima et al., 2008, (111)]

Figure 2.3: Pathways of DSB repair in mammalian cells. D-NHEJ (left) and HRR (right)

2.3.3 DNA-PK dependent non-homologous end-joining (D-NHEJ)

In contrast to HRR, D-NHEJ requires little or no homology to repair DSBs. This pathway is not only used to repair DSBs generated by exogenous DNA-damaging agents, such as IR, but is also required to process the DSB intermediates generated during V(D)J recombination (113). NHEJ can lead to the loss of nucleotides, chromosomal translocations or chromosome fusions. The important proteins involved in D-NHEJ are the KU heterodimer consisting of the Ku70 and Ku80 subunits, catalytic subunit of DNA-PK, DNA-PKcs, Artemis and the complex between Ligase4 (Lig4) and XRCC4 (see Figure 2.3, page 16). Ku heterodimer has a high affinity for DNA ends, which indicates its early role in the NHEJ process.

After induction of DSBs, the Ku70/80 dimer binds to both broken ends of DNA within seconds (114). This DNA-Ku complex recruits the catalytic subunit of DNA-PK (DNA-PKcs). The recruitment of DNA-PKcs to the broken ends increase its kinase activity

almost 10 folds. Once bound to the DNA ends DNAP-Kcs subsequently phosphorylates several cellular target proteins, including p53, the KU polypeptides and itself. In general, IR does not produce clean ligatable DNA ends, but often the ligation step is directly impossible due to the generation of altered nucleotides. Several enzymes including nucleases (Artemis) and polymerases (pol μ and pol λ) are thought to be involved in the processing of single-stranded overhangs before ligation. A complex that consists of DNA, Lig4 and XRCC4 (X-ray-repair-cross-complementing defective repair in Chinese hamster mutant 4) accomplishes the final ligation step. The activity of Lig4/XRCC4 complex is thought to be enhanced by the newly discovered protein XLF/Cernunnos (115).

Both Ku70/80 subunits and DNA-PKcs are essential for repair by NHEJ, although defects in DNA-PKcs generally confer a milder phenotype than defects in Ku. DNA-PK activity is undetectable in Ku-defective cell lines, indicating that DNA binding by the Ku70/80 heterodimer is essential for its activation (113). ATM functions in the NHEJ pathway through the phosphorylation of Artemis and DNA-PKcs (52,116). These results strengthen the importance of ATM in NHEJ. ATM is also shown to be important for V(D)J recombination (117). Moreover, there is a strong correlation between the protein level of DNA-PKcs and ATM. Results with knockdown of DNA-PKcs using siRNA show that the level of ATM is also decreased proportionally to the efficiency of DNA-PKcs knockdown.

It is still unclear to what extent the D-NHEJ pathway contributes to the inaccurate repair of DNA DSBs. After the generation of a DSB by RAG1/2, NHEJ proteins in the V(D)J recombination “inaccurately” join the DNA ends to generate diversity (118). One of the reasons why NHEJ is thought to be error prone relates to the chemical structure of radiation induced DNA ends that precludes end joining from being an accurate repair mechanism. This is further exaggerated by the fact that NHEJ is non-templated repair mechanism, and thus conceptually unable to restore sequence at the damage site. Indeed, the majority of IR induced DSBs have non-ligatable ends and must be subjected to additional processing by a nuclease or polymerase to enable the subsequent ligation reaction. This end processing could result in a loss of several bases adjacent to the break point but also to the addition of new untemplated nucleotides. Mutants with defects in core D-NHEJ proteins remove a large proportion

of DSBs from their genome, utilizing an alternative pathway of NHEJ that operates as a backup pathway [B-NHEJ] (119) that is described next.

2.3.4 DNA-PK independent non-homologous end-joining (B-NHEJ)

Cells with mutations in components of D-NHEJ are still capable of repairing the majority of the IR-induced DSBs utilizing an alternative process, which, surprisingly, is not sensitive to mutations in HRR genes (120,121). This pathway is a distinct form of end-joining, normally suppressed by D-NHEJ, but which can be used as a backup when D-NHEJ is inactivated hence, B-NHEJ. B-NHEJ is likely to be an evolutionarily older pathway with less optimized synapsis mechanisms that rejoins DNA ends with slower kinetics and with half-times of 2-10 h. The slow kinetics and sub-optimal synapsis mechanisms of B-NHEJ allow more time for exchanges through the joining of incorrect ends and cause the formation of chromosome aberrations in wild type and D-NHEJ mutant cells. Within the D-NHEJ repair process, DNA ends are quickly captured by Ku cooperating with DNA-PKcs (see section 2.3.3) and are directed to D-NHEJ for rapid joining, kinetically suppressing the slower backup pathway of B-NHEJ. Alternative pathways of end-joining such as B-NHEJ are expected to contribute significantly to the overall repair of DSBs when components of D-NHEJ are either absent from the vicinity of the break, or genetically/chemically compromised, and may therefore contribute to genome maintenance and stability (122).

Despite the potential consequences of B-NHEJ function, little is known about the underlying mechanism, its regulation, as well as its integration into the cellular DSB-processing apparatus. However, DNA LigIII is implicated in the B-NHEJ and the repair module PARP-1/XRCC1/DNA-LigIII that is involved in the repair of single strand breaks (SSB), also contributes to B-NHEJ (123,124). Interestingly, recent work identifies histone H1 as an additional factor contributing to DSB repair as a component of B-NHEJ (125).

2.4 Chromatin remodelling and DSB repair

The primary function of chromatin is to compress and compact genomic DNA, to protect it from nucleases and to organize genomic DNA into functional compartments. If such a condensed chromatin structure is stably maintained, DNA

metabolic events, such as transcription, replication and DNA repair, may not occur in a proper manner because of the limited accessibility of any enzymatic factors to DNA. The highly ordered structure of eukaryotic chromatin must be appropriately altered to permit access of repair factors to DNA. These alterations are termed chromatin remodelling and are executed by specific remodelling complexes in conjunction with histone modifications. During chromatin remodelling reactions, histones are altered by acquiring post-translational modifications and recruit proteins which can produce conformational changes in chromatin. Chromatin remodelling is achieved through the modification of DNA-histone interactions, removal of histones from DNA, exchanges of pre-existing histones with new histones, and by regulating the local density of histones by sliding them along the nucleosomes (126). Post translational modifications, such as methylation, acetylation, phosphorylation and ubiquitination alter or mark histones to make them available for the subsequent chromatin remodelling reactions. Therefore, these histone modifications are thought to function as a histone code (127). INO80, RSC, SWI/SNF and the SWR complex are some known chromatin remodellers in yeast. Because deficiencies in chromatin remodelling factors result in hypersensitivity to genotoxic agents, chromatin remodelling is considered necessary for the NHEJ and HRR (126,128).

Studies show functional interactions between the chromatin remodelling process and the ATM/MRN complex (36,129-131). These works suggest that ATM and NBS1 are especially critical proteins, which operate during DSB repair through chromatin remodelling. It was found that proteins involved in the HRR pathway are often ubiquitinated and this seems to be essential for HRR. The loss of Ubc13 reduces focus formation of RPA, BRCA1 and Rad51, but not that of γH2AX, or autophosphorylated ATM. These results suggest that Ubc13 is required for the formation of a single-stranded overhang that is essential for the assembly of Rad51 at DSB ends (132). The ubiquitin-proteasome system plays a critical role in HRR-mediated DSB repair (133). Heterochromatic DSBs are generally repaired slowly than euchromatic DSBs, and ATM signalling is specifically required for DSB repair within heterochromatin. About 15% of DSBs require ATM signalling for their repair (134).

2.5 DSB repair pathway selection in different cell cycle phases

The selection of pathway for DSB repair is a major issue in the field. Very little is known in general about the way cells chose between HRR and NHEJ. Is there simply a direct competition between the Ku heterodimer, Rad50/Mre11, or members of the hRad52 pathway in which the winner determines the course of repair? Or there are more subtle means of signalling which help the cell chose the appropriate repair pathway? It would make sense if repair by HRR was activated in late S or G2 phases of the cell cycle when a homologous copy is present in the form of a sister chromatid. How might this be achieved? Is this one of the functions of cell cycle replication forks, or of key members of the hRad52 pathway? Detailed investigation of the signalling events which accompany the formation of DSBs in mammalian DNA is a clear priority (113).

Esashi et *al.* described cell cycle-regulated phosphorylation of BRCA2 that disrupts its interaction with RAD51. This posttranslational modification limits the function of RAD51 and thus HRR in S and G2 (135). It was shown that the phosphorylation of ABCDE and PQR clusters of DNA-PKcs are important determinants of pathway selection in different phases of the cell cycle. The phosphorylated PQR inhibits HRR while inhibition of its phosphorylation promotes HRR (136). Recently it was shown that HRR is increased in kinase dead DNA-PKcs mutants in comparison of null mutant. This phosphorylation related HRR selection is shown to be coordinated with ATM (137). BRCA1 is shown to be required for retention of RPA at the sites of DSBs and the cell cycle-dependent complex formation of BRCA1, CtIP, and MRN contributes to the activation of HR-mediated DSB repair in the S and G2 phases of the cell cycle (87).

In G0 and G1 phase, DSBs are not extensively resected; allowing ATM activation and DSB repair by HRR is largely suppressed. In contrast, in S/G2 phase cells, DSBs are resected, thereby allowing ATR activation and HRR. Notably, this resection and events based on the ensuing ssDNA production take place effectively only after ATM activation has occurred and require the activities of MRN and CtIP. In S/G2 phase, resection provides the ssDNA substrate for HRR and also leads to ATR activation that, among other things, also promotes HRR. Consistent with this idea, *CHK1* has been shown to facilitate HRR, at least in part by mediating

phosphorylation of the key HRR protein, Rad51 (82). NHEJ and HRR are not necessarily independent, since the coordinated action of both pathways is invoked by the cell in order to repair a DSB with minimal error (138). Studies in hamster cells suggested that when NHEJ is impaired, HRR seems to increase and vice versa (139,140). Cells obtained from DNA-PKCs deficient SCID mice that are impaired in NHEJ and V(D)J recombination show normal and even compensatory levels of HRR (141).

It has been demonstrated that HRR using homologous chromosomes does not operate in mammalian cells for repair of DSBs by employing two model systems, namely lymphocytes from Down's syndrome patients with three copies of chromosome 21 and Chinese hamster cells carrying one or two copies of human chromosome 8 (142,143).

CtIP is required not only for repair of DSBs by homologous recombination in S/G2 phase but also for microhomology-mediated end joining (MMEJ) in G1. The function of CtIP in HRR, but not MMEJ, is dependent on the phosphorylation of serine residue 327 and recruitment of BRCA1 (92). The data in this paper support a model in which phosphorylation of Serine 327 of CtIP, as cells enter S phase, and the recruitment of BRCA1 functions as a molecular switch to shift the balance of DSB repair from error-prone DNA end-joining to error-free homologous recombination.

NHEJ substrates are double-stranded ends that have undergone limited processing, whereas HRR substrates are 3' single-stranded tails produced by extensive 5'-end resection. This 5'-end resection is irreversible, at least in yeast, which makes it a good mechanistic stage for the repair pathway choice that channels DSB repair to HRR. DSB-end resection has been demonstrated to be tightly regulated through the cell cycle (144). In mammalian cells, the role of HRR in repair of DSBs is not evident unless the primary repair pathway, NHEJ is non-functional. Mitomycin-C resistance in DNA-PK null cells compared with WT cells suggests that the HRR pathway may be more efficient in cross-link repair in the absence of NHEJ. The incorrectly repaired chromatid damage observed in double-mutant cells may result from failed recombination or another error-prone repair process that is apparent in the absence of the two primary repair pathways (141).

2.6 Repair deficiency, genomic instability and cancer predisposition

Enhanced sensitivity to IR in repair deficient cells was first shown in the mid-1970s and has been found to be a consistent feature of cells from all AT individuals tested (145). Deficient repair of DSBs, resulting in an abnormally high frequency of chromatid breaks after *G*2 exposure of cells to radiation, appears to be associated with cancer predisposition. Un-repaired DSBs contribute to genomic instability. Un-repaired chromatid breaks representing DSBs can result in the chromosome deletions, translocations and gene amplifications seen in human cancers. This cytogenetic response of cells to radiation may be useful as a marker of cancer susceptibility and in identifying individuals at risk of developing cancer in cancer families (146).

The mechanisms controlling G2 radiosensitivity are not yet fully understood (147). It is generally suggested that this sensitivity is determined by HRR, since this process is known to be involved in DSB repair, when cells are in the G2-phase (146). There is a need to understand the relationship between chromatid break frequency and cancer susceptibility, and to identify the genes of low penetrance that control these processes (148). The elevated chromatid radiosensitivity of breast cancer cases is indicative of the presence of low penetrance cancer-predisposing genes (149). It has been proposed that failure to repair a DSB, or its illegitimate repair, may contribute to a cell's progression towards malignancy (150). Defects in HRR cause genomic instability. When the instability leads to aberrant expression or regulation of tumor suppressors or oncogenes, cell transformation and cancer may ensue (88). Clues that implicate HRR in the maintenance of genomic stability and in the prevention of carcinogenesis have come from cells and animals that are deficient in the BRCA1 and BRCA2 genes. A striking connection between HRR and tumorigenesis has been suggested by the observation that the gene products disrupted in these hereditary breast cancer genes interact with the Rad51 protein. The interaction between BRCA2 and Rad51 was found to be direct, while BRCA1 interacts indirectly with Rad51 probably through BRCA2 (151,152). The demonstration that BRCA1-deficient mouse cells are impaired for the repair of chromosomal DSBs by HRR (153), provides evidence that loss of this DNA repair pathway promotes tumorigenesis. Recently the

Rad51 paralog, Rad51C, was shown to be a human cancer susceptibility gene associated with germline mutations in breast and ovarian cancer pedigrees (154).
Fanconi anemia (FA) is a rare hereditary disorder characterized by progressive bone marrow failure, compromised genomic stability and increased incidence of cancer (155). However the roles played by the 13 different FA proteins identified till now is largely unknown, except for the case of FANCD1/BRCA2, which regulates the central HRR protein Rad51 (156). Yamamoto et *al.* reported that FA pathway is required for normal HRR repair (157,158). Recently the biallelic germline mutation in HRR protein Rad51C is shown to be associated with a FA-like syndrome (159).

2.7 Relative roles of repair pathways in DSB repair

The relative contribution of HRR and NHEJ in IR induced DSB repair is controversial in higher eukaryotes (160). Genetic models deficient in important factors of either pathway show increased radiosensitivity (161). The radiosensitivity of HRR deficient cells is mainly found in the late-S/G2 phase of the cell cycle (22,162). The disruption of Rad52 confers no sensitization in mammalian cells (163,164). Inactivation of Rad54 causes a modest increase in radiosensitivity that is mainly associated with the late S/G2 phase (165). The disruption of Rad51 is lethal (166), but mutations in the Rad51 paralogs *XRCC2* and *XRCC3* confer significant radio-sensitivity (167,168). The relatively high radio-resistance of NHEJ-defective mutants in the late S/G2 portion of the cell cycle further suggests that HRR promotes survival when sister chromatids are present (169,170). The DNA-PKcs and Ku80 deficient cells also showed increased radio-sensitivity (171,172). These results clearly implicate the importance of both D-NHEJ and HRR at the cell survival level.
On the other hand, the results of DSB-rejoining by pulsed-field gel electrophoresis (PFGE) show that while D-NHEJ is the main repair pathway in mammalian cells, HRR deficient cells show no DSB repair defect even in the G2 phase of the cell cycle. In the absence of D-NHEJ, HRR does not seem to play a detectable role in DSB repair; rather B-NHEJ appears to repair DSBs in these cells with slower kinetics, and its function is shown to be enhanced in the G2-phase of the cell cycle (173-175).

Much of our understanding of mammalian HRR is derived from a system that utilizes the rare cutting endonuclease I-SceI to generate a site-specific DSB (176). The I-SceI system clearly demonstrates that site directed chromosomal DSBs are potent inducers of HRR (177,178). I-SceI induced DSBs are useful for identifying proteins that influence HRR efficiency and/or outcome, but they do not fully mimic IR-induced DSBs which must be processed to produce ligatable/extendable ends, nor other damage produced by IR including single-strand breaks, base damage, and complex lesions. In mammalian cells, approximately 30-50% of I-SceI endonuclease induced site directed chromosome breaks have shown to be repaired by homologous recombination, while the rest is repaired by NHEJ (177).

Golding et *al.* has reported that ATM is an important regulator of HRR and suggests that the effect of caffeine on HRR is primarily attributable to the inhibition of ATM, rather than ATR or any other PI3KK. They concluded that HRR is important for DSB repair in glioma cells with overall contribution to repair between 2 and 30% with the remaining repair carried out primarily by NHEJ. Also ATM specifically regulates HRR, whereas NHEJ is not influenced by ATM manipulation (179). Evans et *al.* has shown that HRR plays a very important role in the repair of Tirapazamine induced DSBs in human and hamster cells (180). Riballo et *al.* showed that ATM along with Artemis is required for repair of a sub-fraction of DSBs (about 10%) rejoining through alternative NHEJ, that requires Artemis nuclease activity, H2AX, 53BP1, Nbs1, Mre11, and DNA-PK (116).

It is known that radiation induced cell killing results from the induction of chromosomal aberrations (CAs). DSBs are the primary lesions which give rise to chromosome aberrations (9,10). The chromosome changes we observe at metaphase are the end-product of a chain of events initiated by a molecular lesion in the DNA, particularly the DSB (181). Broken chromosomes are a serious threat to cell survival and their repair is necessary for the maintenance of genomic integrity. This repair of broken chromosomes is a challenge for dividing cells that need to distribute equally their genetic information to the daughter cells. Unresolved DSBs appear as chromosome breaks that can be efficiently scored in conventional metaphases or G2-premature chromosome condensation (G2-PCC). Evidence from genetically engineered mammalian cells containing an I-SceI DSB site shows that a single DSB in a cell's genome is sufficient to cause a chromatid break (182).

It has been considered by some authors (146) that the disappearance of chromatid breaks with time after irradiation represents DSB rejoining (based on the breakage first model).

What is the contribution of HRR in the processing of IR induced DNA DSBs and how can it affect the yield of chromosomal aberrations? The importance of HRR in the maintenance of genomic integrity is not questionable, but the exact mechanism of its involvement in protecting cells from IR induced chromosomal aberrations remains elusive (Bedford, JS, ISCA, 2009). The results of IR induced DSB repair obtained at different end-points like rejoining of DSB repair and cell survival point to a discrepancy regarding the contribution of HRR that requires elucidation.

2.8 The goals of the present thesis

The aim of the project is to study the relative contribution of D-NHEJ and HRR to the repair of G2-chromosomal breaks and to investigate the role of these repair pathways in IR induced G2-checkpoint signalling.
The experiments are designed in such a way that DSB induction and repair are studied through their transformation to chromosome breaks that can be visualized by cytogenetic approaches in the G2 and M-phases of the cell cycle. The assay used allows the measurement of repair in a specific phase of the cell cycle without the need for synchronization and at very low radiation doses. This is not easily possible with other techniques such as PFGE, or the comet assay etc.

The assay employed here is different from the classical “G2-assay” in terms of the post irradiation time interval chosen to collect the samples. In the “G2-assay”, chromosomal-radiosensitvity is evaluated at a fixed time point (146,147,183,184), while in this study kinetics of chromatid breaks is followed by collecting irradiated cells at hourly intervals for up-to 5 h post irradiation. This approach has been used in the past and the repair of chromatid breaks was shown to be connected with the repair of a subclass of DSBs (185,186).

Appropriate Chinese hamster, mouse and human mutants/knockout cells are employed to study the role of D-NHEJ and HRR in the repair of chromatid breaks. Experiments are also carried out with cells deficient in important DDR signalling proteins to check the connection between DDR signalling and repair of chromatid

breaks. The results are confirmed by employing specific and non-specific inhibitors of DSB repair and DDR signalling proteins.

The second important aspect of this thesis is to find the possible connection between HRR and G2-checkpoint signalling. Flow cytometric analysis is employed to study G2-checkpoint activation, maintenance and recovery in HRR and NHEJ deficient Chinese hamster and human cell lines. Histone H3 gets phosphorylated at serine-10 early in mitosis and, therefore an antibody against phospho-histone H3 can be used to assess the IR-induced G2 checkpoint delay or entry into mitosis (187). Mitotic index (MI) variations are studied using this technique after exposure of cells to 1 Gy X-rays. These variations in mitotic index reflect the G2-checkpoint activation and subsequent recovery.

3. Materials and Methods

3.1 Cell lines

Table 3.1 The cell lines used for the experiments are listed in the table below

S/No	Species	Cell line name	Cell type	Mutation(m)/Knockout (KO)
1	Chinese hamster	CHO10B4	Ovarian	Wild type
2	Chinese hamster	V79-S171	Fibroblast	Wild type
3	Chinese hamster	Irs1SF	Ovarian	XRCC3 m
4	Chinese hamster	Irs1tor	Fibroblast	XRCC2 m
5	Chinese hamster	Irs1-clone1	Fibroblast	Corrected for XRCC2
6	Chinese hamster	Xrs5	Ovarian	XRCC5 (Ku80) m
7	Chinese hamster	Irs20	Ovarian	DNA-PKcs m
8	Chinese hamster	Irs20-K147E	Ovarian	Corrected for DNA-PKcs
9	Chinese hamster	XR1	Ovarian	XRCC4 m
10	Chinese hamster	Rad51D1	Ovarian	Rad51D KO

11	**Chinese hamster**	**Irs1SF Clone X5-1**	**Ovarian**	**Corrected for XRCC3**
12	**Chinese hamster**	**Irs2 tor**	**Fibroblast**	**Rad51B m**
13	**Chinese hamster**	**Irs3 tor**	**Fibroblast**	**Rad51C m**
14	**Chinese hamster**	**V-C8**	**Fibroblast**	**BRCA2 m**
15	**Mouse**	**MEF Ligase 4+/+ p53 -/-**	**Mouse embryo fibroblasts**	**Wild type**
16	**Mouse**	**MEF Ligase 4-/- p53 -/-**	**Mouse embryo fibroblasts**	**Ligase 4 KO**
17	**Mouse**	**MEF Ligase 4+/+ / RAD54-/- / p53 -/- 347E Clone 1**	**Mouse embryo fibroblasts**	**Rad54KO**
18	**Mouse**	**MEF Ligase 4-/- / RAD54-/- / p53 -/- 346B**	**Mouse embryo fibroblasts**	**Ligase 4 and Rad54 double KO**
19	**Mouse**	**H2AX -/-, p53 -/-**	**Fibroblasts**	**H2AX KO**
20	**Mouse**	**H2AX +/+ p53 -/- (PW)**	**Fibroblasts**	**Control for H2AX KO**
21	**Mouse**	**53BP1-/- ES Cells**	**Embryonic stem cells**	**53BP1 KO**
22	**Mouse**	**53BP1+/+ ES Cells**	**Embryonic stem cells**	**Control for 53BP1 KO**
23	**Human**	**M059K**	**Glioma**	**Control for M059J**
24	**Human**	**M059J**	**Glioma**	**DNA-PKcs m**
25	**Human**	**HCT116 wt**	**Colorectal tumor**	**Wild type**
26	**Human**	**HCT116 DNA-PKcs -/-**	**Colorectal tumor**	**DNA-PKcs KO**
27	**Human**	**HCT116 Ligase 4 -/-**	**Colorectal tumor**	**Ligase 4 KO**

28	**Human**	**Capan-1,BHA Transfection**	**Breast**	**Control for capan1**
29	**Human**	**Capan1**	**Pancreatic tumor**	**BRCA2 m**
30	**Human**	**HCC4.10 (HCC1937 with wt BRCA1)**	**Breast cancer**	**Control for HCC4.12**
31	**Human**	**HCC4.12 (HCC1937 with empty vector)**	**Breast cancer**	**BRCA1 m**
32	**Human**	**Mrc5 SV1**	**Fibroblasts**	**Wild type**
33	**Human**	**AT5BIVA**	**Fibroblasts**	**ATM m**
34	**Human**	**Nbs1LB**	**Fibroblasts**	**NBS1 m**
35	**Human**	**GM-847**	**Fibroblasts**	**Control for GM847-ATRkd**
36	**Human**	**GM-847ATRkd**	**Fibroblasts**	**ATR kinase dead (doxycycline induced)**
37	**Chinese hamster**	**V3**	**Ovarian**	**DNA-PKcs m**

3.2 Cell culture and growth conditions

All cells were incubated at 37°C in an atmosphere with 5% CO_2 and 95% air (MCO-18AIC, Sanyo CO_2 incubator). CHO10B4 were grown in McCoy's 5A medium supplemented with 5%FBS and all other hamster cells were grown in MEM supplemented with 10% FBS. Mouse embryo fibroblasts (MEF) were grown in DMEM medium supplemented with 10% FBS and antibiotics. Human HCT116 colorectal tumor cells were grown in McCoy's 5A medium supplemented with 10% FBS and antibiotics. Breast cancer and pancreatic tumor cells were grown in RPMI medium supplemented with 15% FBS and antibiotics. Human glioma cells were maintained in

DMEM with 10% FBS. The rest of human fibroblast cells were grown in MEM medium supplemented with 10% FBS and antibiotics.

3.3 Chemicals and inhibitors

Colcemid (L-6221, Biochrome AG) was used at 0.1 µg/ml to accumulate metaphases (Stock: 10µg/ml in PBS w/o Ca^{++}, Mg^{++}). For G2-PCC induction, Calyculin A (C-3987, LC laboratories) was used at the concentration of 50-100nM (Stock: 10µM in DMSO). Calyculin A is a Serine/Threonine Phosphatase Inhibitor, which induces PCCs efficiently in interphase cells (188). Hypotonic solution was prepared by dissolving Potassium Chloride (Carl Roth GmbH & Co.) in Milli-Q water (Z00QSV001, Millipore, 18.2 MΩ·cm at 25°C) to obtain 75mM working solution. Carnoy's fixative was prepared by adding 3 parts methanol (Sigma Aldrich) and 1 part glacial acetic acid (Carl Roth GmbH & Co.). Hypotonic solution and Carnoy's fixative were prepared fresh on the day of the experiment. Microscope slides, 76X26 mm (H-872, Roth Karlsruhe) were used for making cytogenetic preparations. 2.5 ml of ready to use Giemsa stain (Carl Roth GmbH & Co.) was diluted in 50 ml of Sorenson's buffer (10582-013, Gibco, Invitrogen) to stain the slides. Slides were mounted using 24X60 mm coverslips (H-878, Roth Karlsruhe). Entellan (Merck) was used as a mounting medium. Caffeine (C-8960 Sigma-Aldrich) was dissolved in Milli-Q water to prepare a 200mM stock solution. The solution was sterilized by passing through a 0.22µm filter and stored at RT. 4mM caffeine was used as the working concentration. Low concentrations of caffeine are shown to inhibit the kinase activity of ATM kinase, and higher concentrations also inhibit ATR and DNA-PKcs (189). 2-morpholin-4-yl-6-thianthren-1-yl-pyran-4-one (118500, Calbiochem, also known as KU-55933) was dissolved in DMSO (Dimethyl sulfoxide) (D8418, Sigma-Aldrich) to prepare a 10mM stock solution that was stored at -20 °C in the dark. 10 µM was used as the working concentration. KU-55933 is a highly specific small molecule ATP competitive inhibitor of ATM. It was identified via screening of a drug library based on LY294002, a non-specific inhibitor of ATM (190). PCI-24781 (Mr 434) was obtained from Pharmacyclics. PCI-24781 (formerly CRA-024781) is a broad-spectrum phenyl hydroxamic acid HDAC inhibitor being evaluated in phase I clinical trials in patients with neoplastic disease. Adimoolam et *al.* (2007) have reported that PCI-24781 radio-sensitizes the human and hamster cells by down-regulating Rad51 dependent HRR

(112). 2 μM and 0.5 μM PCI-24781 were used as the working concentrations. MIRIN is a specific inhibitor of the MRN complex. It inhibits MRN dependent ATM activation and abolishes the endonuclease activity of Mre11. The consequence of MIRIN-dependent MRN inhibition is the prevention of G2/M checkpoint activation and HRR repair (191). MIRIN (6-(4-hydroxyphenyl)-2-thioxo-2, 3-dihydro-4 (1H) pyrimidinone) was synthesized by Specs Research laboratory, Delft, The Netherlands (ID No: AG-690). 30, 50 and 100 μM MIRIN was used as the working concentration. PARP-1 inhibitor PJ34 was obtained form Alexis Biochem. 10 μM was used as the working concentration

3.4 Irradiation

Irradiations were carried out with an X-ray machine (GE-Healthcare) operated at 320-kV, 10 mA with a 1.65 mm Al filter (effective photon energy approximately 90 kV), at a distance of 50 cm, and a dose rate of approximately 1.3 Gy/min. Dosimetry was performed with a PTW and/or a chemical dosimeter, which were used to calibrate an infield ionization monitor. An even irradiation was ensured by rotating the radiation table. Cells were returned to the incubator immediately after IR.

3.5 Clonogenic survival assay

Cell radiosensitivity to killing was determined by the clonogenic survival assay. Exponentially growing cells were irradiated with different doses, at room temperature and trypsinised immediately at 37°C. Cells were seeded into 60mm dishes, in duplicate, at various densities aiming at approximately 100 colonies/dish. After an incubation period of about 7 days, colonies were stained with crystal violet solution and colonies with more than approximately 50 cells were counted. Cell populations enriched in G1 and G2 phase cells were obtained by centrifugal elutriation and used to determine cell radiosensitivity to killing.

3.6 Cytogenetic assays

The experiments are designed in such a way that DSB induction and repair are studied through their transformation to chromosome breaks that can be visualized by cytogenetic approaches in the G2 and M-phases of the cell cycle. The assay allows measurement in specific phases of the cell cycle and at very low radiation doses,

which is not easily possible with other techniques of DSB repair such as PFGE, the comet assay etc..

3.6.1 Assay to measure the kinetics of G2-chromosome breaks at metaphase:

Exponentially growing cell cultures were irradiated with 1 Gy X-rays. Following irradiation cells were allowed to repair for 1 to 5 h at 37°C to detect only the response of cells in G2 phase at the time of irradiation. Colcemid was added for 1 h aimed to block metaphases prior to harvesting the respective time point, except for the 1 h time point, where colcemid was added for 30 min before harvest to allow the migration of cells which were at the mitosis during irradiation. Cells were trypsinized, treated with hypotonic solution (75mM KCl) for 10 min at room temperature (RT) and fixed in Cornoy's fixative (3X). The fixed cells were dropped on clean glass slide and stained with 3% Giemsa stain prepared in Sorenson's buffer. For each experimental point about 300 cells were scored for chromatid damage from three independent experiments. Standard criteria were used for scoring. During scoring chromatid breaks and gaps were considered, the latter only when longer than the chromatid width. The chromosome exchanges were counted as two chromatid breaks. Bright field microscopy (Olympus VANOX-T, Japan) was employed to facilitate scoring. This protocol employed here is the modified protocol used by Bryant et *al.*, 2008 (186). See appendix 4 and 5 (page 98-99) for the CHO and human metaphase spreads, showing chromatid breaks and chromatid exchanges.

3.6.2 Premature chromosome condensation (PCCs)

To study the influence of G2 checkpoint on chromosome break repair kinetics, exponentially growing cell cultures were irradiated with 1 or 2 Gy X-rays and allowed to repair at 37°C for 1 to 4 h after irradiation. 50 nM Calyculin-A was added for 45 min before harvesting the respective time point to induce PCCs. This time for calyculin-A treatment is considered in the calculation of the repair time. Cells were harvested and prepared for cytogenetic analysis in the similar way as for metaphases. About 150 G2-PCCs were scored for each experimental point from three independent experiments. During scoring excess PCC fragments were considered. The chromosome exchanges were counted as two chromatid breaks.

Bright field microscopy (Olympus VANOX-T, Japan) was employed to facilitate scoring.

3.7 Flow cytometry

3.7.1 Cell cycle analysis by flow cytometry

Propidium iodide (PI) binds to DNA proportional to its mass. Cell cycle distribution was assessed by measuring PI fluorescence on a flow cytometer. Cells were washed with cold PBS and trypsinized at 37°C for 5 min. Single cell suspensions were prepared in 5 ml cold fresh media. About 1 million cells were collected and centrifuged at 1500 RPM, 4°C for 5 min. The cell pellets were washed with cold PBS and fixed in 70% ethanol at -20°C overnight. Supernatant was removed by centrifugation at 1500 RPM for 5 min. Pellets were washed with cold PBS and incubated in PBS containing PI (40 µg/ml) (81845, Sigma-Aldrich) and RNase (62 µg/ml) (R4875, Sigma-Aldrich) at 37°C for 30 min in the dark. Samples were measured on a flow cytometer (COULTER EPICS XL, BECKMAN COULTER) according to pre-established protocols, see Appendix 2 (page 96). 20,000 cells per sample were counted and the single cell population was gated to obtain standard histograms. Histogram files (*.HST) were generated by counting the frequency of cells with same PI signal intensity.

The fractions of cells in the different phases of the cell cycle were calculated using the Wincycle® software. HST files were loaded into the Wincycle®. The parameter “S-phase growing order” was carefully chosen between 0.1 to 2, until the prediction model fitted the histogram shape. Cell cycle distributions were automatically calculated. G2 arrest kinetics was obtained by plotting the G2 fraction as a function of time after IR.

3.7.2 Determination of mitotic index

Bivariate flow cytometry was used to simultaneously measure DNA content and the levels of phosphorylated Histone-3 (H3-pS10). Briefly, 0.6-0.8 million cells were prepared as single cell suspension in cold fresh media after the completion of respective time point. After centrifugation at 1500 RPM, the cell pellets were washed with cold PBS and fixed in 70% ethanol at -20°C overnight. Supernatants were

removed by centrifugation at 1500 RPM for 5 min. Cells were further permeabilized in 2 ml permeabilization solution (PBS + 0.25% Triton X-100) on ice for 15 min. Supernatants were removed by centrifugation at 1500 RPM and the pellets were washed with cold PBS. Cells were blocked in blocking buffer (PBS + 0.05% Tween-20 + 1% BSA (8076.2, ROTH)) at RT for 45 min in the dark with gentle agitation. Primary antibody H3-pS10 rabbit polyclonal (06-570, Upstate) was diluted 1:150 in blocking buffer. After centrifugation at 1500 RPM for 5 min, the pellets were suspended in 100 µl diluted primary antibody and incubated for 2 h at RT in the dark with gentle agitation. The primary antibody was diluted in 5 ml PBS and the cells were washed 3 times with PBS. Secondary antibody Rabbit IgG-FITC (AP307F, Chemicon), was diluted in blocking buffer (1:200). Cell pellets were incubated with 100 µl conjugated secondary antibody for 90 min at RT in the dark. Secondary antibody was removed by diluting in 5 ml PBS and washing with PBS three times. Cells were then incubated with PI plus RNase at 37°C for 30 min in the dark before measuring on a flow cytometer. A total 20,000 cells were measured. Proper gating was applied to detect H3-pS10 positive cells and to calculate their fraction in the total population. Compensation was applied when necessary. See appendix 3 (page 97) for the settings for bivariate flow cytometry.

4. Results

4.1 Clonogenic survival assay

The clonogenic survival assay is a well accepted method for determining the radiosensitivity of different cell lines. We evaluated therefore selected CHO wild type, D-NHEJ and HRR mutants for radiosensitivity to killing. Results are summarised in Figure 4.1 (page 36). Both exponentially growing D-NHEJ (xrs5) and HRR (irs1SF) deficient cells show increased radiosensitivity compared to their wild type counterpart (Figure 4.1 A, page 36). Also HRR deficient irs1 cells showed significant radiosensitivity when compared to V79, the wild type parental cell line (Figure 4.1 B, page 36). These results confirm previous studies (163,167) and show that both HRR and D-NHEJ protect cells of IR-induced cell killing. The above experiments were carried out with asynchronously growing cell populations that contain cells in all phases of the cell cycle. It is well established that the radiosensitivity of cell fluctuates considerably throughout the cell cycle, with cells at mitosis and early S being radiosensitive and cells in mid-G1 and mid-S radioresistant. When measuring intrinsic radiosensitivity using asynchronous cells, the result obtain will reflect the radiosensitivity of the most radioresistant subpopulation i.e. S-phase in the case of CHO cells (161). For a more in depth analysis of this parameter, we checked the radio-sensitivity of elutriated G1, S and G2 cells from CHO and irs1SF cells. No significant difference was observed between G1, S and G2 fractions in CHO cells (Figure 4.1 C, page 36), a result reflecting the suboptimal synchronization achieved. However, in S-phase populations of irs1SF cells a significant increase in radiosensitivity is observed compared to G1 and G2 cell populations (Figure 4.1 D, page 36), which is in line with earlier studies implicating HRR in the development of S-phase dependent radioresistance (192).

4.2 The contribution of D-NHEJ and HRR in the repair of IR-induced G2-chromosome breaks

As outlined in the introduction, DSBs are the principal lesions, responsible for IR induced cell killing, an effect that is mainly mediated through the induction of

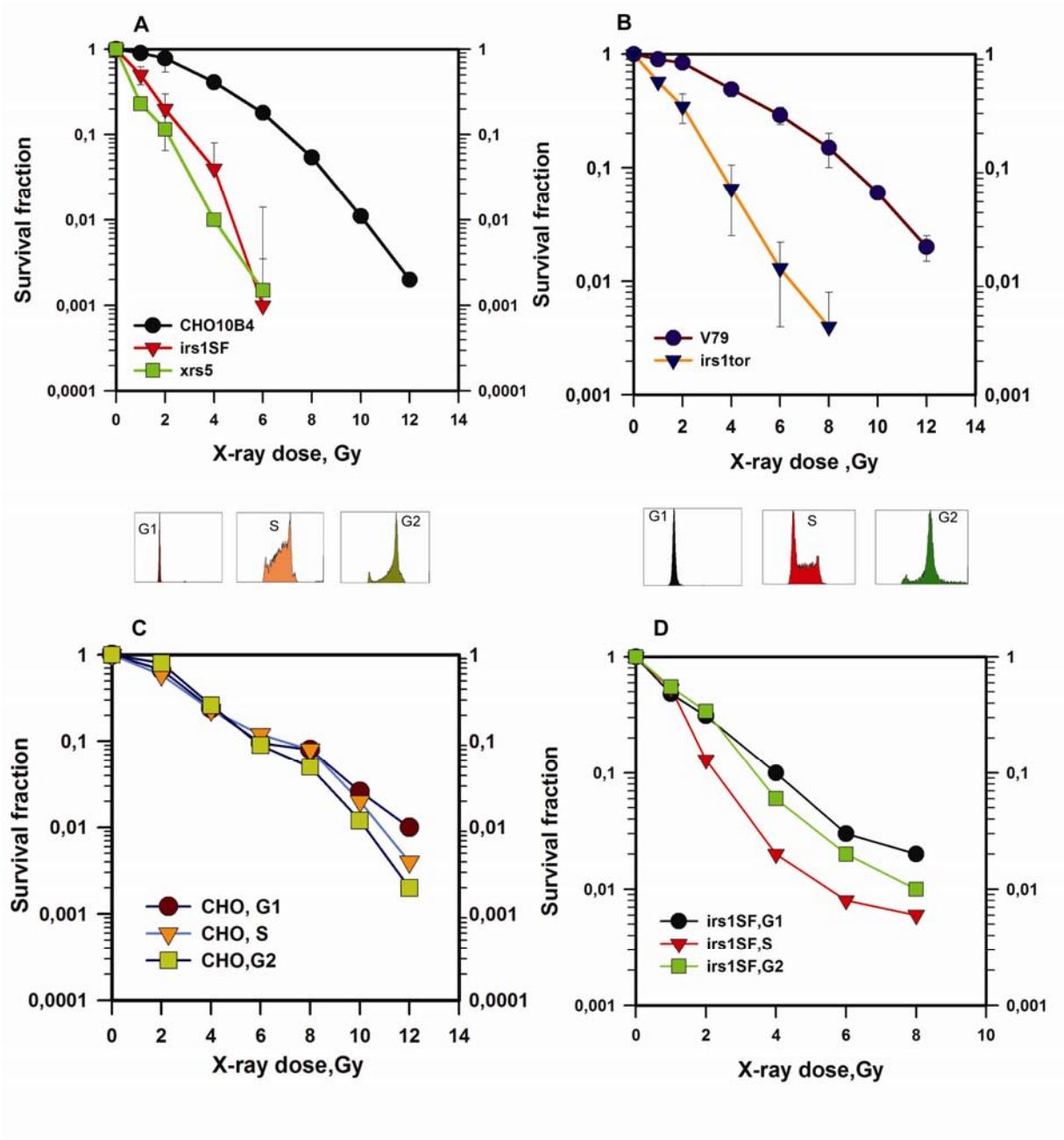


Figure 4.1 Clonogenic survival assay in wild type, D-NHEJ and HRR deficient Chinese hamster cells. Exponentially growing asynchronous cultures, or synchronous cells enriched in G1, S and G2 phase of the cell cycle obtained by centrifugal elutriation, were irradiated with various doses of X-rays and allowed to form colonies. The surviving fraction is plotted as a function of radiation dose. (A) Exponentially growing wild type, D-NHEJ and HRR mutants of Chinese hamster ovary origin, (B) Exponentially growing wild type and HRR mutants of Chinese hamster fibroblast origin, (C) Elutriated CHO wild type cells, (D) Elutriated HRR deficient irs1SF cells. Histograms of elutriated fractions used for experiments are shown in the top part of (C) and (D).

chromosome aberrations. We wished to evaluate the contribution of D-NHEJ and HRR to the repair of DSBs through their transformation into G2-chromosomal breaks (cytogenetically termed as chromatid breaks). The assay employed is described in the "Introduction" and under "Materials and Methods". The kinetics of chromatid break rejoining in wild type, D-NHEJ and HRR deficient Chinese hamster mutants are summarized in Figure 4.2 (page 39). The wild type CHO10B4 and V79 cells show about four chromatid breaks/cell on average, after 1 h of 1 Gy X-rays; these breaks are repaired with time and ~ 0.5 chromatid breaks/cell (~10% of initial breaks) remain unrejoined 5 h post irradiation (Figure 4.2 B, E and G, page 39). This decrease in chromatid breaks follows first order kinetics and represents the repair of a subset of DSBs, which cause chromatid breaks. They represent 10-20% of the induced DSBs and is presently unknown why only a subset of DSBs causes direct chromatid breaks. The half repair time ($T_{1/2}$) of the repair of chromatid breaks in wild type cells is ~ 2 h. In the following sections we compare the results of wild type CHO cells to those obtained with D-NHEJ and HRR deficient mutants. Please refer to Table 4.1 (page 62) for a comparison of residual damage and repair half times between wild type cells and the different mutants.

4.2.1 NHEJ deficiency does not affect the repair of chromosome breaks in the G2- phase of the cell cycle

The D-NHEJ Chinese hamster mutants xrs5, irs20 and XR1 show increased levels of chromatid breaks (~ 2 - 2.5 fold above the WT), which are repaired with kinetics identical to that of wild type cells (Figure 4.2 B, C, D and G, page 39). The $T_{1/2}$ for xrs5, irs20 and XR1 is about 2.5 h, 2 h and 3.3 h respectively and residual damage after 5 h is between 8 and 15% (Figure 4.2 H, page 39). These results indicate that fast repair is one of the reasons why relatively few DSBs lead to chromosome breaks. The repair of these breaks seems to be unaffected by the D-NHEJ deficiency, at least for the cells that somehow manage to enter mitosis despite the activation of the G2 checkpoint. Our results with xrs5 cells confirm the results of earlier studies and validate our methods and approaches (186,193).

4.2.2 Is HRR responsible for the repair of G2-chromosomal breaks?

Since in D-NHEJ deficient cells HRR remains in principle functional, we measured the kinetics of chromatid break repair in HRR deficient CHO cells. We wished to examine whether HRR is responsible for the repair of chromatid breaks seen in D-NHEJ deficient cells. HRR deficient irs1 and irs1SF cells show an elevated number of initial chromatid breaks (~1.5 times more than WT cells). Notably, the majority (~ 80%) of these breaks remain un-rejoined (Figure 4.2 E, F, G and H, page 39). This is particularly striking vis-à-vis the observation that in HRR deficient cells D-NHEJ repairs practically all IR-induced DSBs, even at significantly higher doses of radiation and even for cells tested in the G2 phase of the cell cycle (174,175). Correction of irs1 cells through transfection of a functional *XRCC2* gene (irs1 Clone1 cells) restores their capability to repair G2-chromosome breaks (Figure 4.2 E, page 39). These results identify HRR as the candidate repair pathway for chromosome breaks in the G2-phase of the cell cycle. We also confirm that D-NHEJ defects do not compromise the repair of chromatid breaks in cells escaping the G2 arrest. However, our results with irs1 cells do not support the results reported by Bryant P.E. et *al.*, 2008 (186), where it is claimed that irs1 cells repair G2-chromosome breaks with kinetics similar to the wild type. The reasons for this apparent discrepancy are presently unknown.

4.2.3 Coupled HRR/NHEJ defects cause increase the initial level of chromatid damage and inhibit its repair

The results with irs1 and irs1SF cells suggest a role for HRR in the repair of G2-chromosome breaks. However, B-NHEJ can also contribute to the repair of chromatid breaks in G2 irradiated cells. This expectation is strengthened by the observation that B-NHEJ is more prominent in the G2-phase of the cell cycle (174,175). To explore this possibility we evaluated repair of G2-chromosome breaks in MEF-347B double knockout cells, which are deficient in Lig4 and Rad54 (proteins involved in D-NHEJ and HRR pathways of DSB repair, respectively). Wild type MEFs, *LIG4*$^{-/-}$ MEFs and *Rad54*$^{-/-}$ MEFs are used as controls in this experiment. Exponentially growing cells were irradiated with 1Gy X-rays and prepared for scoring chromatid breaks.

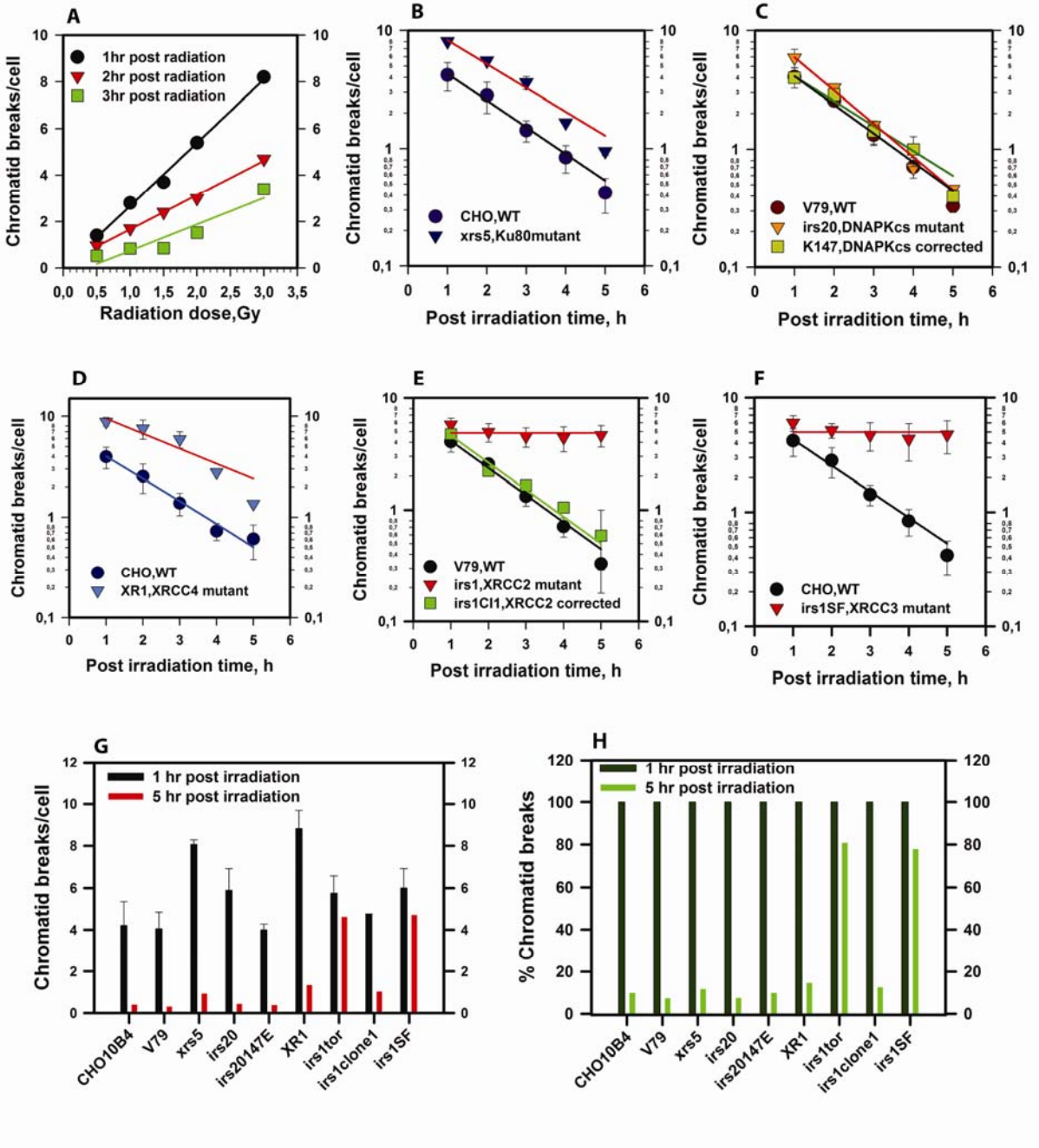


Figure 4.2 Kinetics of chromatid breaks in exponentially growing Chinese hamster cells, irradiated with 1 Gy X-rays and allowed to repair for up-to 5 h after irradiation. The number of chromatid breaks per cell is plotted against time. (A) Dose response curves for CHO cells, (B, C and D) repair kinetics in D-NHEJ deficient hamster cells, (E and F) HRR deficient hamster cells, (G) Comparison between initial and residual damage in different hamster mutants, (H) Comparison of % residual damage [% of initial breaks] in different hamster mutants. The data points represent mean and standard-deviation from 2 or 3 independent experiments.

The results obtained in this experiment are summarized in Figure 4.3 (page 41). *LIG4*$^{-/-}$ MEFs show an ~2 fold increase in initial chromatid breaks, which are repaired in cells that escape the G2 arrest with kinetics similar to that of wild type cells - $T_{1/2}$ ~ 3 h (Figure 4.3 A, F and G, page 41). *Rad54*$^{-/-}$ MEFs 346E show about same number of initial chromatid breaks as wild type cells but leave ~ 52% breaks un-rejoined at the 5 h time point; the $T_{1/2}$ in this set is about 4.5 h (Figure 4.3 B, F and G, page 41). The double knockout *LIG4*$^{-/-}$*/Rad54*$^{-/-}$ (347B) MEFs show an ~ 3 fold increase in initial chromatid breaks and leave their majority (~ 71%) un-rejoined at the 5 h time point (Figure 4.3 C, F and G, page 41). Another interesting observation in this experiment is that the frequency of chromatid exchanges increased ~ 5 fold in *LIG4*$^{-/-}$*/Rad54*$^{-/-}$ (347B) MEFs double-knockout cells in comparison of WT cells (Figure 4.3 E, page 41). Chromatid exchanges (Figure 4.3 D, page 41, indicated by arrow-head) are chromosome miss-rejoining events. Please refer to Table 4.2 (page 63) for a comparison of residual damage and half times of repair between wild type and mutant cells.

The above results strengthen and support results obtained with HRR deficient CHO cells and suggest a role for HRR in the repair of G2-chormosomal breaks in rodent cells. The results with *LIG4*$^{-/-}$ MEFs also allow similar conclusions to those obtained with CHO D-NHEJ mutants. *Rad54*$^{-/-}$ MEFs do not show as a strong phenotype as the HRR deficient CHO mutants, both in terms of initial damage and the kinetics of rejoining of chromatid breaks. This difference is likely due to the less essential role of Rad54 in HRR as compared to XRCC2 and XRCC3. Indeed previous studies showed that disruption of *Rad54* in the mouse confers a modest increase in radiosensitivity to killing (165), while *XRCC2* and *XRCC3* mutants display significant radiosensitivity (167,168).

The many fold increase in chromatid exchanges in *LIG4*$^{-/-}$*/Rad54*$^{-/-}$ MEFs, suggests that the error-prone alternative pathway, B-NHEJ, rejoins chromatid breaks in the absence of the two main repair pathways. However, the observation that chromatid break repair was practically completely inhibited in HRR mutants suggests that exchanges occurred between DSBs that are not normally transformed to chromosome breaks. Thus an increase of miss-rejoining events in the form of chromatid exchanges is observed when an error free pathway like HRR is

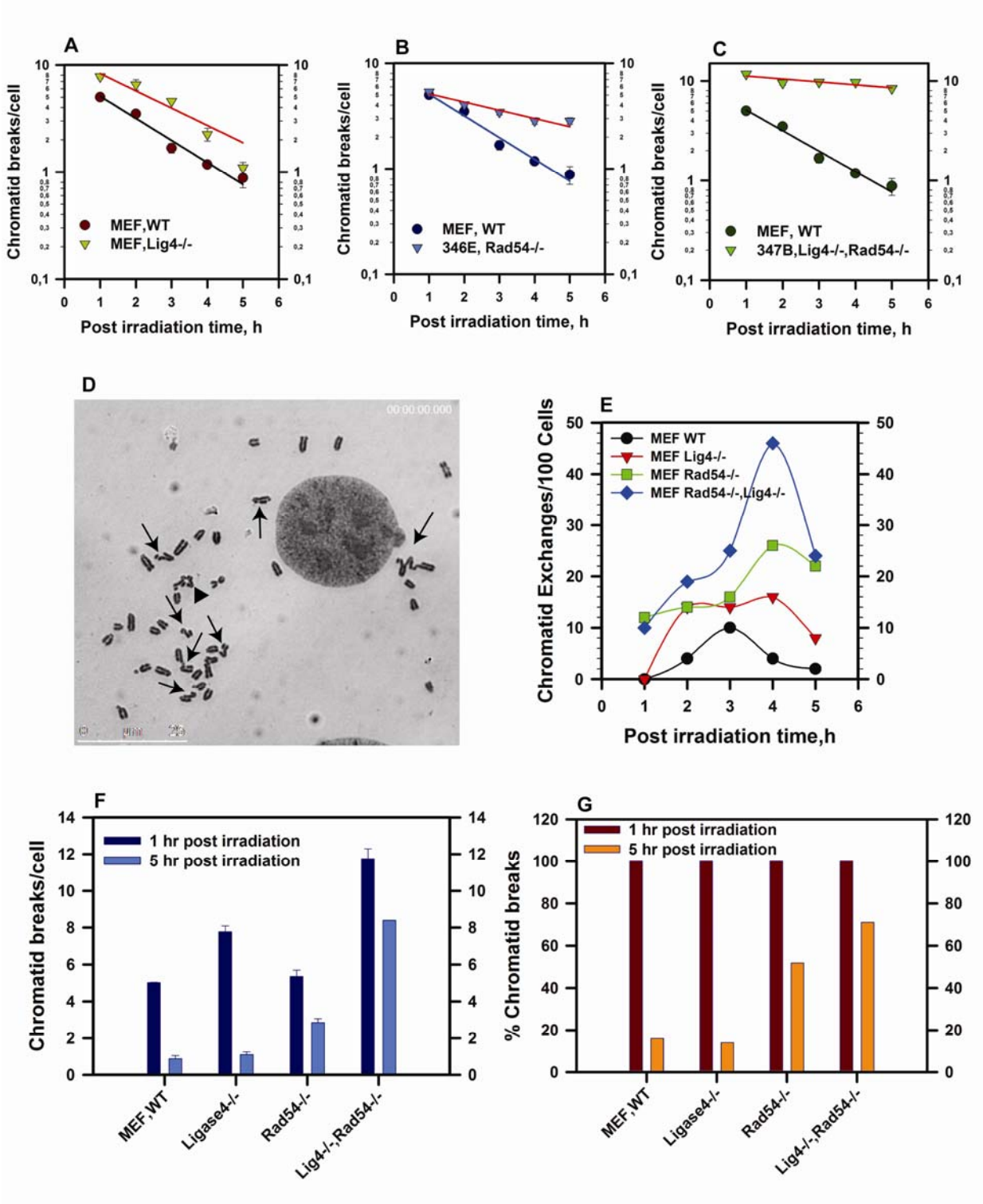


Figure 4.3 Kinetics of chromatid breaks and exchanges in exponentially growing MEFs irradiated with 1 Gy X-rays and allowed to repair for up-to 5 h after irradiation. Number of chromatid-breaks/cell is plotted against time. (A) *LIG4*$^{-/-}$ cells, (B) *Rad54*$^{-/-}$ cells, (C) *LIG4*$^{-/-}$ *Rad54*$^{-/-}$ double knockout cells, (D) Metaphase spread; arrows represent chromatid breaks and arrow head represents chromatid exchange, (E) Kinetics of chromatid exchanges/100cells, (F) Comparison between initial and residual damage in different MEF mutants, (G) Comparison of % residual damage (% of initial breaks) in different MEF mutants.

compromised. Pluth et *al.* in 2001 also observed incorrectly repaired chromatid damage in cells with defects in both D-NHEJ and HRR, which they attributed to failed homologous recombination or to the function of another error-prone repair process operating in the absence of the two primary repair pathways (141). Notably, *LIG4*$^{-/-}$ */Rad54*$^{-/-}$ (347B) MEFs show no defect for IR induced DSB-rejoining over that observed in *LIG4*$^{-/-}$ MEFs, even when analyzed in the G2 phase of the cell cycle (174).

4.2.4 Is HRR also important for the repair of G2-chromosomal breaks in human cells?

The next important question was whether species specific variability exists for HRR mediated repair of G2-chromosome breaks? To address this question, we studied the kinetics of chromatid break repair in wild type, D-NHEJ and HRR mutant cells of human origin. Exponentially growing cells were irradiated with 1 Gy X-rays and prepared for analysis of chromatid break repair kinetics. The results obtained are summarized in Figure 4.4 (page 43). The wild type human cells show ~ 6 to 8 chromatid breaks as initial damage, which is repaired with a $T_{1/2}$ of 2 to 3 h in cells from different origins (Figure 4.4 A, B, C and D, page 43). The residual damage in wild type cells from different origins ranges between ~ 2.5% to 20% (Figure 4.4 F, page 43). In *LIG4*$^{-/-}$ or *DNA-PKcs*$^{-/-}$ HCT116 colon cancer cells, an ~ 2 and 3 fold increase in the initial number of chromatid breaks is observed, respectively, as compared with wild type cells (Figure 4.4 A, page 43). The kinetics of chromatid breaks is identical to that of wild type cells and the majority of breaks are repaired within 5 h (residual damage of ~ 30% and $T_{1/2}$ ~ 3.2 to 4 h). The *DNA-PKcs* mutant human glioma cells, M059J, show an ~ 2.5 fold increase in initial damage with the majority of breaks being repaired within 5 h (~ 22% residual damage) but with slower kinetics ($T_{1/2}$ ~ 3.5 h) than wild type cells ($T_{1/2}$ ~ 1.7 h) [Figure 4.4 B on page 43, also see table 4.3 on page 64]. BRCA2 mutant pancreatic human tumor cells Capan 1 show no remarkable increase in the initial damage, but the majority of chromatid breaks (~ 80%) remains un-rejoined (Figure 4.4 C, E and F, page 43). On the other hand, BRCA1 mutant breast cancer human cells HCC4.12 show ~ 1.5 fold increase in the initial damage and leave ~ 85% chromatid breaks un-rejoined (Figure 4.4 D, E

and F, page 43). Refer to Table 4.3 (page 64) for a comparison of residual damage and of the repair half times between wild type and mutant human cells.

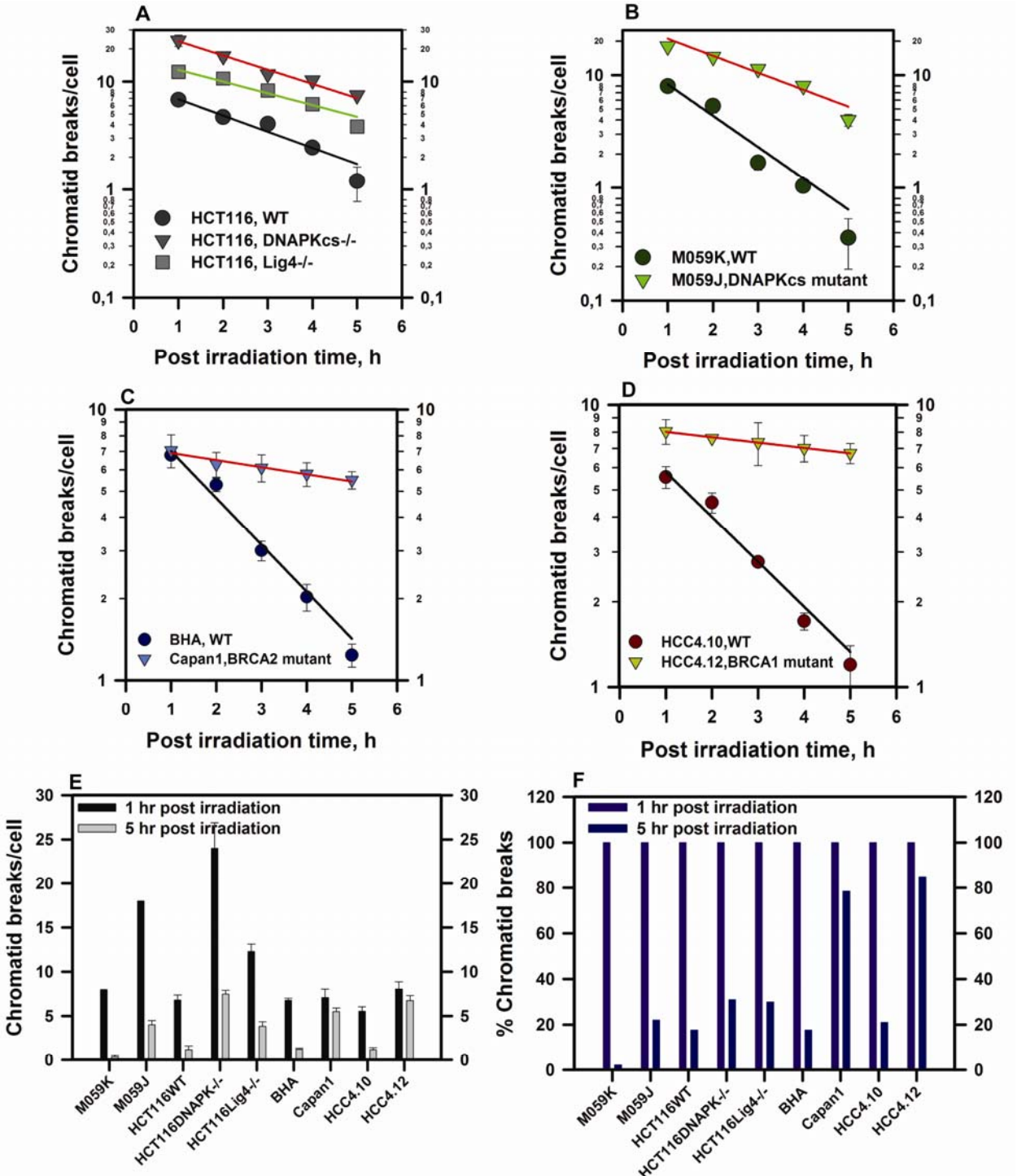


Figure 4.4 Kinetics of chromatid breaks in exponentially growing human cells, irradiated with 1 Gy X-rays and allowed to repair for up-to 5 h after irradiation. The number of chromatid breaks/cell is plotted against time. (A) D-NHEJ deficient colon cancer cells, (B) DNA-PKcs deficient glioma cells, (C) BRCA2 deficient pancreatic tumor cells, (D) BRCA1 deficient breast cancer cells, (E) Comparison between initial and residual chromosome damage in different human mutants, (F) Comparison of % residual damage (% of initial breaks) in different human mutants.

These results show that wild type human cells are more sensitive for X-rays than wild type rodent cells (initial number of breaks ~ 6 to 8/Gy for the former than ~ 4/Gy for the latter). The results obtained with D-NHEJ and HRR deficient human mutants are qualitatively similar to those obtained for rodent mutants. Thus, no species specific variability is observed for this HRR dependent repair of G2-chromosomal breaks. Interestingly BRCA1 deficient cells show as a strong phenotype as BRCA2 deficient cells, despite the fact that BRCA1 is thought to be involved in HRR only indirectly and possibly by reinforcing DSB generated signalling (194). The results in aggregate emphasize the importance of breast cancer susceptibility genes in HRR mediated repair of G2-chromosomal breaks.

4.2.5 Chemical down regulation of Rad51 inhibits the repair of G2-chromosomal breaks in wild type hamster and human cells

Rad51 is a key protein of HRR taking part in the homology search and synapsis that follows the end preparation step (see Figure 2.3, page 16). Rad51 deficiency is lethal and as a result no Rad51 mutants or knockout cells or animals are available for genetic studies. PCI-24781 is an HDAC-inhibitor, which radiosensitizes tumor cells and is being tested clinically for application in treatment of human cancer. Adimoolam et *al.* (2007) have shown that PCI-24781 radiosensitizes human colon cancer and Chinese hamster cells via inhibition of HRR through down-regulation of Rad51 protein (112).

As HRR deficient hamster and human mutant cells show remarkable defects in the repair of G2-chromosomal breaks; we decided to treat selected hamster and human wild type and HRR mutants with PCI-24781 for 24 hours before exposure to 1 Gy X-rays. The purpose of this experiment was to investigate whether PCI-24781 mediated inhibition of HRR affects the kinetics of rejoining of chromatid breaks? Exponentially growing CHO (wild type) and irs1SF cells are treated with 2 µM and breast cancer BHA (corrected cells) are treated with 0.5 µM PCI-24781 for 24 hours before irradiation. The results are summarized in Figure 4.5 (page 45). CHO cells show strong inhibition of chromatid break repair (~ 60% residual damage) when treated with PCI-24781 before irradiation; while cells which are only irradiated can repair the majority of these breaks (~ 10% residual damage). HRR deficient irs1SF cells, on the other hand show no significant difference in chromatid break repair when treated with

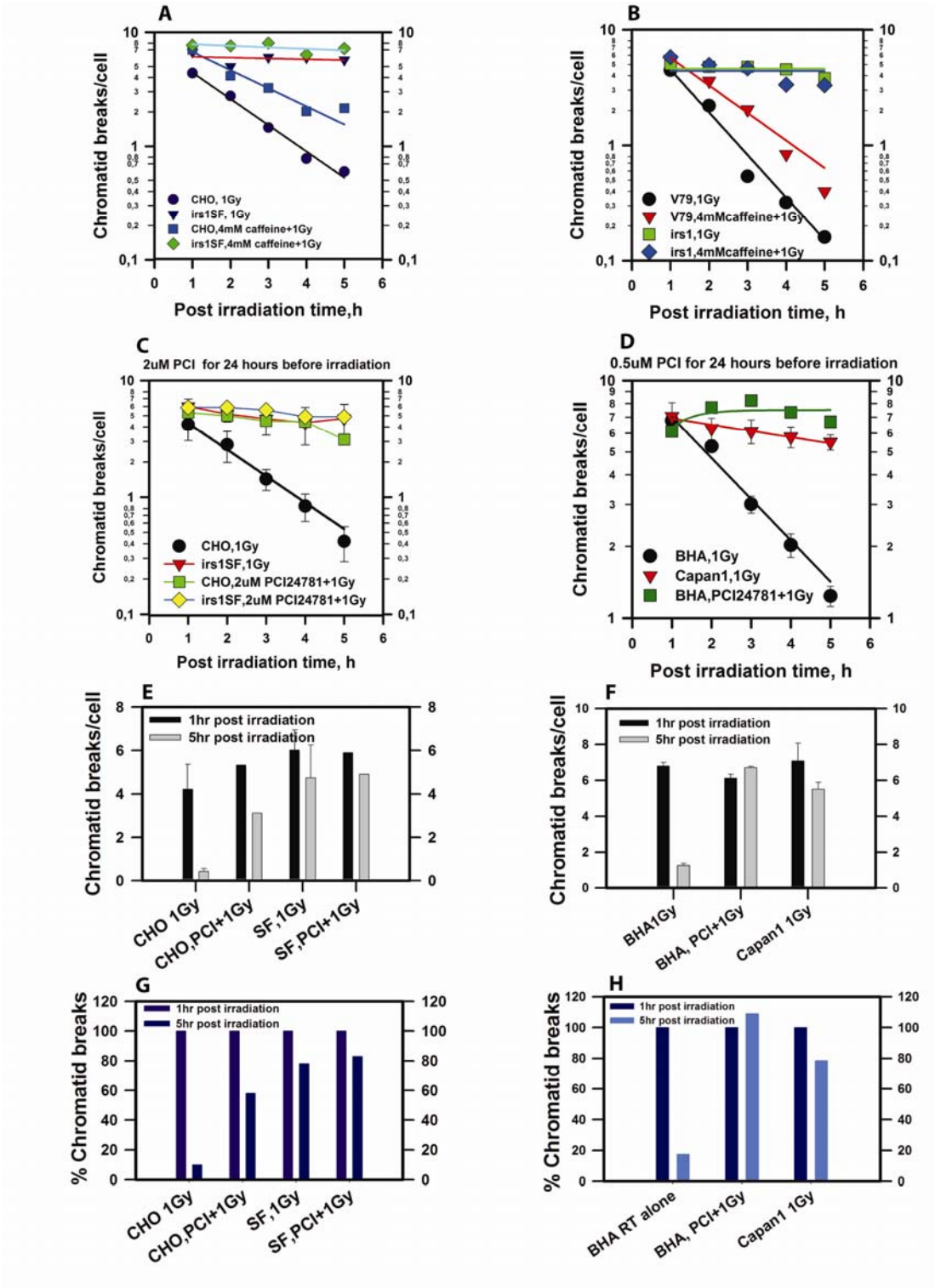


Figure 4.5 Effect of different inhibitors on the kinetics of chromatid breaks in exponentially growing Chinese hamster and human mutants. Cells were treated with the indicated concentrations of caffeine for 1 h and PCI-24781 for 24 h before 1 Gy X-rays and allowed to repair for 5 h. Drugs were maintained in the cultures during the entire postirradiation incubation period. (A and B) Effect of 4 mM caffeine on wild type and HRR deficient Chinese hamster mutants, (C, E and G) Effect of HDAC inhibitor PCI-24781 on wild type and HRR deficient hamster mutant, (D, F and H) Effect of HDAC inhibitor PCI-24781 on BHA human cells.

PCI-24781 in comparison of those which are only irradiated (Figure 4.5 C, E and G, page 45,). Human BHA cells also show nearly complete inhibition of chromatid break repair when irradiated after treatment with PCI-24781 (Figure 4.5 D, F and H, page 45).

These results confirm that inhibition of HRR, even using chemical means (PCI-24781), abrogates chromatid break repair in G2.

4.3 Role of DDR proteins in the repair of G2-chromosomal breaks

DDR signalling and DSB repair pathways are thought to act in a well coordinated manner to detect and process IR induced DNA damage. The next question we wished to address was whether important DDR proteins contribute directly to the repair of G2-chromosomal breaks? To address this question, we employed mouse and human mutants defective in key components of DDR. We also employed for this purpose specific and non-specific chemical inhibitors. The results are summarized in Figure 4.6 and Figure 4.7 (page 48 and page 52 respectively).

4.3.1 H2AX and 53BP1 defects do not affect the repair of G2-chormosomal breaks

H2AX and 53BP1 are early DNA damage sensing proteins, which contribute to the mounting of the DDR signalling cascade. To examine the role of these proteins on chromatid break repair kinetics we used mouse knockout cell lines. Exponentially growing wild type, *H2AX*$^{-/-}$ and *53BP1*$^{-/-}$ mouse cells are irradiated with 1 Gy X-rays and the kinetics of chromatid break repair is determined. Both *H2AX*$^{-/-}$ (Figure 4.6 A, page 48) and *53BP1*$^{-/-}$ (Figure 4.6B, page 48) cells repair chromatid breaks with kinetics similar to those of their wild type counterparts.

4.3.2 The role of DNA-PK, ATM and ATR in the repair of G2-chromosomal breaks

DNA-PKcs, ATM and ATR belong to the kinase family PI3KK and have been clearly implicated in DDR signalling. These proteins are considered as the most important players in G2-checkpoint response and in the maintenance of genomic stability. While DNA-PKcs is one of the central components of D-NHEJ, ATM and ATR are

thought to be principally involved in DDR signalling. To investigate the role of these proteins in the repair of chromatid breaks we used the DNA-PKcs deficient human glioma cells, M059J, the ATM mutant human fibroblasts, AT5BIVA, and the doxycycline inducible ATR kinase dead GM847 human cells. Results with M059J cells are described in detail in section 4.2.4. (page 42) Here it is important to mention that these cells repair chromatid breaks with slower kinetics than other D-NHEJ mutants (compare Figure 4.4 A and B, page 43). AT5BIVA cells show significant but not complete inhibition of chromatid break repair. The initial damage is ~ 2 fold higher than wild type cells and the % residual damage is ~ 45% with $T_{1/2}$ ~ 4.2 h; the $T_{1/2}$ for wild type cells is ~ 1.7 h (Figure 4.6 F on page 48 and Table 4.4 on page 65). Interestingly ATR kinase dead cells show almost complete inhibition of chromatid break repair with ~ 93% of residual damage after 5 h of irradiation (Figure 4.6 G, H, I on page 48 and Table 4.4 page 65).

4.3.3 Nbs1 and BRCA1 defects significantly inhibit G2-chromosomal break repair

Both Nbs1 and BRCA1 proteins have been shown to be important in DDR signalling. They are also implicated in HRR mediated signalling. The results with BRCA1 deficient breast cancer cells are also discussed in section 4.2.4 (page 42), where it is shown that the repair of chromatid breaks is severely compromised in these cells (also see Figure 4.6 D, page 48). Nbs1 deficient human fibroblasts also show significant inhibition of chromatid break repair with ~ 62% residual damage left after 5 h of irradiation (Figure 4.6 C, H and I, page 48). These results suggest that proteins involved in DDR signalling show remarkable defects in the repair of G2-chromosomal breaks.

Please refer to Table 4.4 (page 65) for a comparison of residual damage and half times of repair between wild type and DDR signalling mutants.

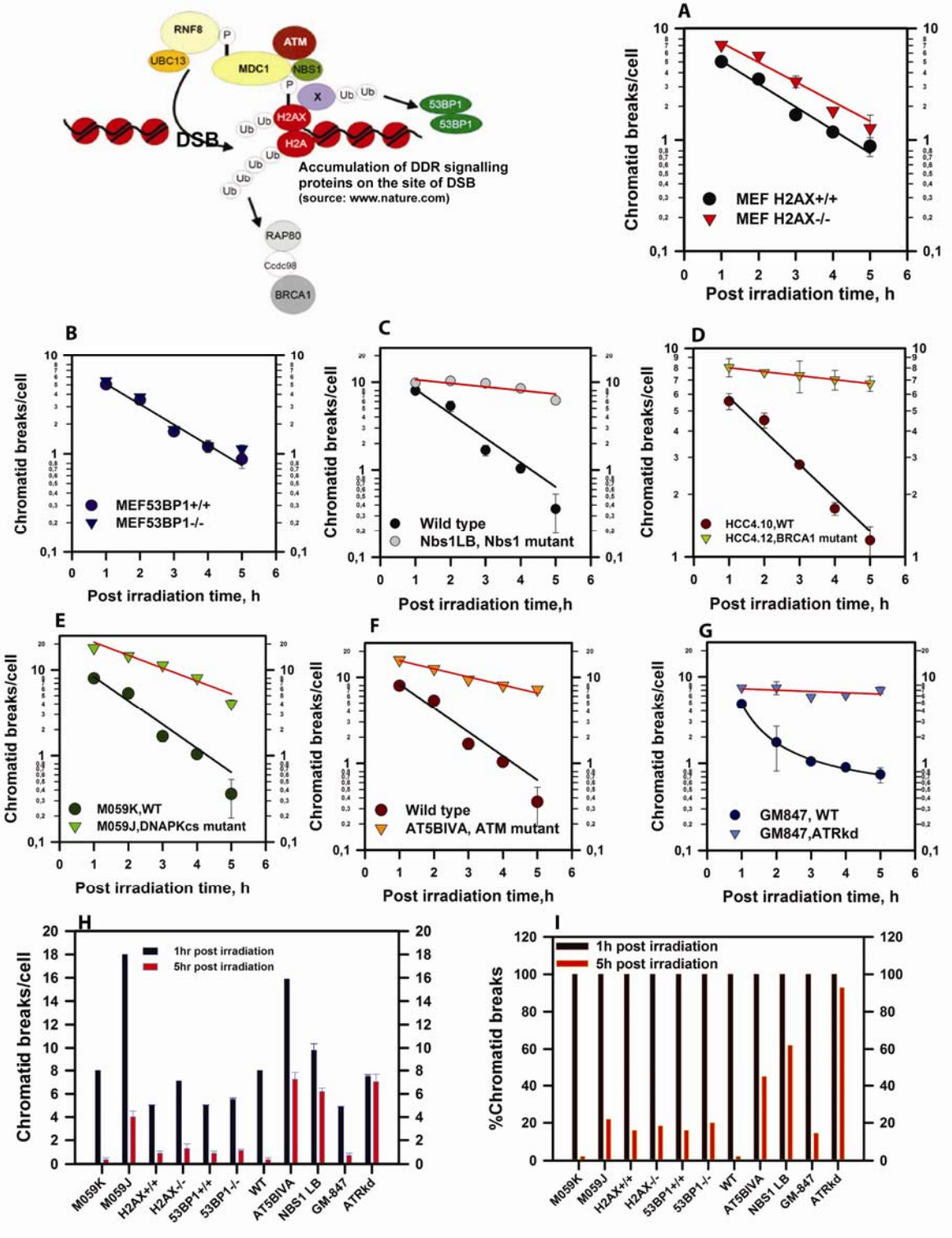


Figure 4.6 Kinetics of chromatid breaks in exponentially growing mammalian cells deficient in DDR-signalling proteins. Cells are irradiated with 1 Gy X-rays and allowed to repair for up-to 5 h. The number of chromatid breaks/cell is plotted against time. (A) *H2AX*$^{-/-}$ MEFs, (B) *53BP1*$^{-/-}$ MEFs, (C) *Nbs1* deficient human fibroblasts, (D) *BRCA1* deficient breast cancer cells, (E) *DNA-PKcs* deficient human glioma cells, (F) Human *ATM* mutant, (G) Doxycycline-induced *ATR* kinase dead human cells, (H) Comparison between initial and residual chromosome damage, (I) Comparison of % residual damage (% of initial breaks).

4.3.4 Transitional knock down of CtIP completely inhibits repair of G2-chromosomal breaks in M059J cells

CtIP, along with BRCA1 and MRE11, promotes DSB repair through HRR by contributing to DNA end resection that produces the recombinogenic 3' single stranded DNA tails (91,92). CtIP interacts with the MRN complex and enhances MRE11's endo-nuclease activity for ssDNA, indicating that MRN functions at the initiation step of HRR by promoting DSB end resection through its interaction with CtIP. In this experiment we transitionally knocked down CtIP with the help of siRNA in DNA-PKcs deficient M059J cells. Cells in which CtIP had been knocked down show complete inhibition of chromatid break repair with ~ 98% residual damage at 5 h post irradiation (Figure 4.7D on page 52 and Table 4.5 on page 66).

4.3.5 Chemical inhibition of MRE11 inhibits repair of G2-chromosomal breaks in D-NHEJ deficient cells

LIG4$^{-/-}$ HCT116 cells were treated with 30, 50 and 100 µM of MIRIN (MRE11 inhibitor) for 1 h before 1 Gy of X-rays. Only irradiated cells are taken as control for this experiment. As shown in Figure 4.7 E (page 52), MIRIN inhibits the repair of chromatid breaks significantly in a concentration independent manner.

4.3.6 Effect of caffeine on the repair of G2-chromosomal breaks

The results presented above clearly show that ATR plays a very important role in HRR mediated repair of G2-chromosomal breaks, while ATM deficiency shows a less pronounced effect. DNA-PKcs deficient human glioma cells show normal but slow repair of chromatid breaks. We were interested to further look into the role of ATM and ATR in the repair of chromatid breaks using caffeine. Caffeine has been shown to radiosensitize hamster cells by targeting HRR (195). It is also known to inhibit ATM, ATR and DNA-PKcs in vitro (189), and is therefore considered a non-specific inhibitor of signalling proteins of the PI3KK family. In this experiment we examined the effect of caffeine on the kinetics of chromatid break repair in selected wild type, HRR and D-NHEJ mutants of hamster and human origin. Exponentially growing cells were treated with 4mM caffeine for 1 h before 1 Gy X-rays and prepared for cytogenetic analysis following different post irradiation incubation times. While no

additional effect of caffeine is observed on the kinetics of chromatid break repair in HRR deficient irs1 and irs1SF cells, the repair is partially inhibited in wild type CHO and V79 cells (Figure 4.5 A and B, page 45). Caffeine inhibits the repair of chromatid breaks almost completely (~ 80% residual damage) in the DNA-PKcs deficient human glioma cells M059J (Figure 4.7 A, F and G, page 52), while the wild type M059K cells show partial repair inhibition (Figure 4.7 A, H and I, page 52). Results with caffeine-treated irs1 and irs1SF cells are in agreement with the hypothesis that caffeine inhibits HRR and this is why an additional effect is not observed. Results with DNA-PKcs deficient M059J cells suggest that almost complete inhibition of chromatid break repair in these cells is due to the inhibition of ATM and ATR. It should be noted that low levels of ATM are reported in M059J cells (196,197). M059K cells show only partial inhibition of chromatid break repair when treated with 4 mM caffeine before irradiation, which suggests that either caffeine does not inhibit HRR completely at this dose, or that B-NHEJ repairs these breaks. To check this possibility, we treated cells with the PARP-1 inhibitor PJ34. PARP-1 is a candidate component of B-NHEJ. M059J and M059K cells were treated with a combination of 10 μM PJ34 (PARP1-inhibitor) and 4mM caffeine for 1 h before exposure to 1 Gy X-rays. No additional effect of PJ34 is observed on the kinetics of chromatid breaks in either cell line (Figure 4.7 F, G, H and I, page 52).

4.3.7 Effect of a specific ATM inhibitor on the repair of G2-chromosomal breaks

Caffeine is a non-specific inhibitor of PI3K kinase family. To explore the exact role of the ATM kinase in the repair of chromatid breaks in human glioma and colon cancer cells, we used the specific ATM inhibitor, KU55933. Exponentially growing cells were treated with 10 μM KU55933 for 1 h before exposure to 1 Gy X-rays. The inhibitor was maintained in the cultures during the entire period of repair incubation. The kinetics of chromatid break repair is studied for up-to 5 h post irradiation. KU55933 significantly inhibits repair of chromatid breaks with ~ 80% residual damage in M059J cells and partially in M059K cells with ~ 20% residual damage (Figure 4.7 B on page 52 and Table 4.5 on page 66).

KU55933 also inhibits repair of chromatid breaks in DNA-PKcs knockout human HCT116 colon cancer cells (~ 67% residual damage), but has no effect on wild type

HCT116 cells (Figure 4.7 C on page 52 and Table 4.5 on page 66). These results suggest that although the ATM levels are low in M059J cells, these low levels are enough to signal and support repair of the majority of chromatid breaks in the presence of a functional ATR. Specific (KU5933) or non-specific (caffeine) inhibition of ATM significantly compromises repair in these cells. The same applies also to HCT116 DNAPK$^{-/-}$ cells, which are shown to have low levels of ATM (Mladenov E., unpublished data). The results with M059K cells suggest that inhibition of ATM alone can not inhibit repair of chromatid breaks completely, which emphasizes on the importance of ATR in this response. We have no explanation for the results obtained with HCT116 wild type cells.

4.4 Does the G2-checkpoint play a role in the repair of G2-chromosomal breaks?

DSBs are potent inducers of cell cycle checkpoints mediated through DDR signalling. G2-M checkpoint activation delays/stops cell division to facilitate repair. The G2-checkpoint is the last chance to correct genomic damage prior to cell division. DDR signalling proteins of PI3K kinase family, DNA-PKcs, ATM and ATR have been shown to be the key players in the activation of this checkpoint. In the cytogenetic assay described before, chromatid breaks are scored at metaphase, which means that irradiated G2-cells are allowed to progress through the cell cycle and to reach metaphase and that during this process cells are under the influence of the G2-checkpoint. Assuming that the G2-checkpoint stops cell cycle progression and facilitate the repair of DSBs, the question arises as how and why HRR deficient cells leave the majority of chromosome breaks unrepaired? To study the influence of G2-checkpoint on the repair of G2-chromosomal breaks we decided to perform calyculin-A induced premature chromosome condensation (PCC) in wild type and HRR mutants of Chinese hamster and human origin. We also checked the G2-checkpoint activation, maintenance and recovery employing flow cytometric methods in wild type, D-NHEJ and HRR mutants to systematically address this issue. The results obtained are described in the following sub-sections.

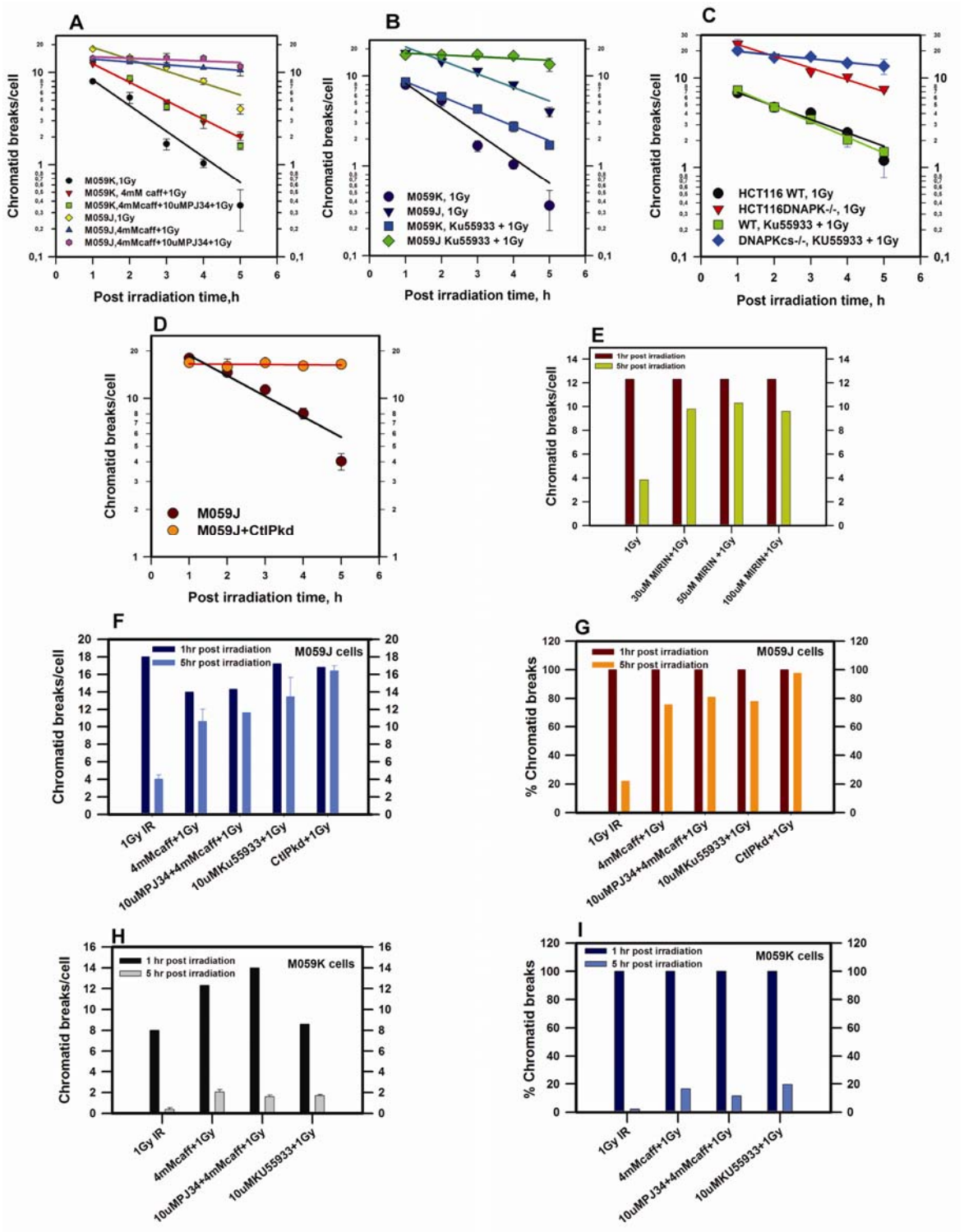


Figure 4.7 Effect of different inhibitors on the kinetics of chromatid breaks in wild type and NHEJ deficient human cells. Exponentially growing cells were treated with the indicated concentrations of different inhibitors, alone or in combination, for 1 h before exposure to 1 Gy of X-rays. Drugs were maintained in the cultures for the entire period of the experiment. Treated cells were allowed to repair for 5 h. Chromatid breaks/cell is plotted against time. (A) Effect of caffeine and PJ34 on DNA-PKcs deficient glioma cells, (B and C) Effect of ATM inhibitor KU55933 on DNA-PKcs deficient human glioma and colon cancer cells, (D) Effect of transitional CtIP knockdown on DNA-PKcs deficient glioma cells, (E) Effect of mirin on *LIG4*$^{-/-}$ colon cancer cells,(F and H) Comparison between initial and residual damage in M059J and K cells after different treatments, (G and I) Comparison of % residual damage (% of initial breaks) in M059 J and K cells after different treatments.

4.4.1 The repair of G2-chromosomal breaks is influenced by the G2 checkpoint

Calyculin-A induces condensation of chromatin in interphase cells. A great advantage of this approach is that damage can be visualized and scored directly in G2 cells, which eliminates the influence of the G2 checkpoint on repair. G2-PCCs can easily be distinguished from metaphase chromosomes as they lack the centromeric constrictions (Figure 4.8 and 4.9 A, B, C, and D, page 54). In this experiment exponentially growing wild type and HRR mutants are irradiated with 1 or 2 Gy X-rays and G2-PCC break kinetics measured for up to 4 h post irradiation. The results of CHO cells are shown in Figure 4.8 (page 54). Surprisingly, the initial level of damage is found similar in both WT and HRR deficient cells. While wild type cells repair normally, the majority of breaks are left un-rejoined in HRR deficient cells (Figure 4.8 E and F, page 54).

Wild type and HRR deficient human cells also show the same level of initial induction of chromosome breaks, but compromised repair in the presence of HRR defects (Figure 4.9 E and F, page 55). BRCA2 deficient Capan1 cells show excessive damage when irradiated with 2 Gy. Therefore, to facilitate scoring we irradiate also with 1 Gy. The results of PCC measurements confirm our results obtained with metaphase chromosome break analysis showing the importance of functional HRR in the repair of IR induced G2-chromosomal breaks. The similar level of damage induction and the different kinetics of rejoining in WT and HRR mutants suggest that the G2 checkpoint is playing an important role in this repair process and probably works inefficiently in HRR deficient cells. To address the latter possibility we examined systematically G2-checkpoint activation and recovery in wild type and repair deficient cells, which is discussed in the next sections.

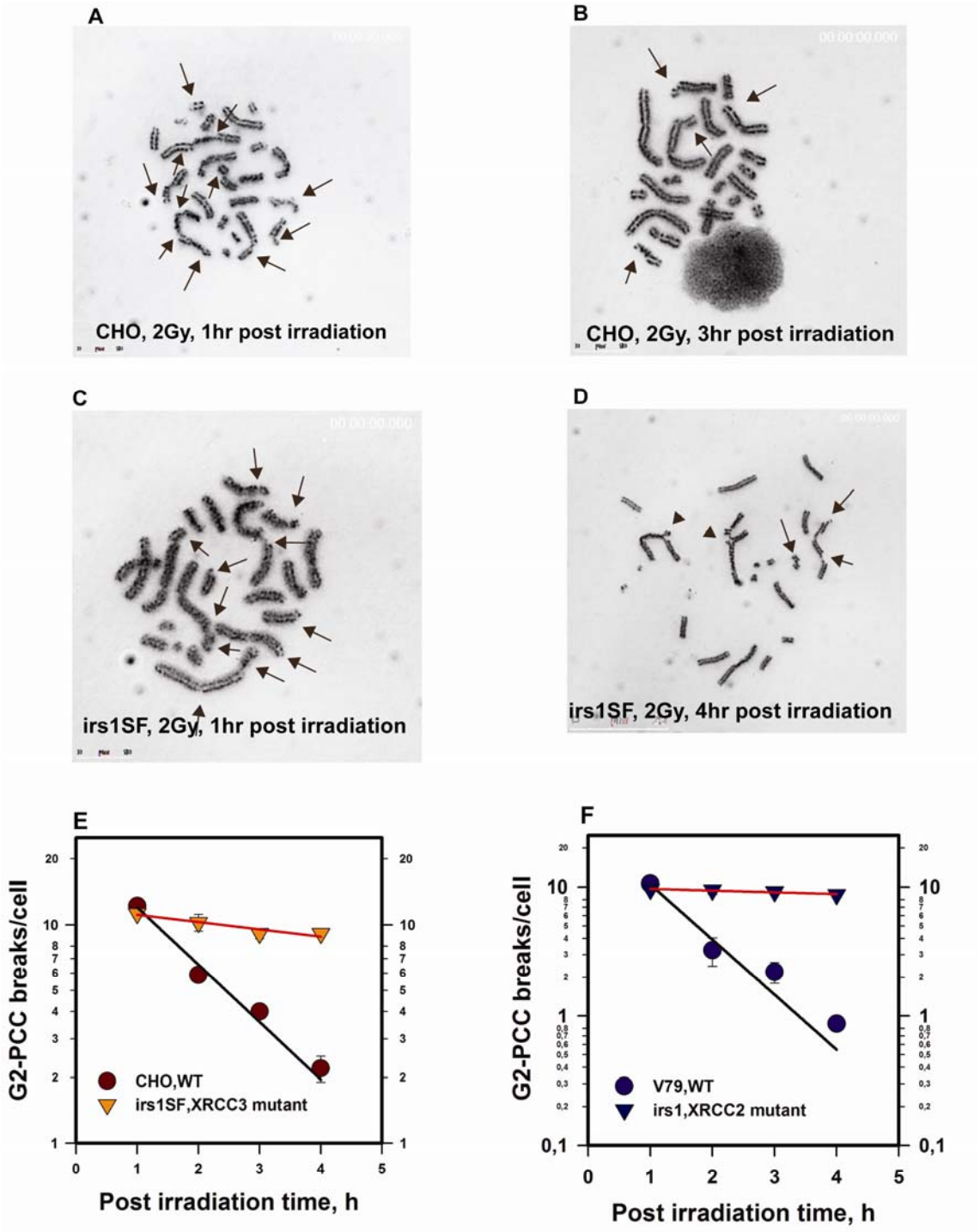


Figure 4.8 Kinetics of calyculin-A induced G2-PCC breaks in exponentially growing wild type and HRR-deficient Chinese hamster mutants irradiated with 2 Gy X-rays. Cells were allowed to repair for up-to 4 h. G2-PCCs were induced by adding 50 nM calyculin A for 45 minutes and this time is included in the repair time. G2-PCC breaks/cell is plotted against time. (A, B, C and D) Calyculin A induced G2-PCC breaks in CHO and HRR deficient irs1SF cells at different post irradiation times; arrows represent PCC-breaks and arrow heads represent chromatid exchanges, (E and F) Kinetics of G2-PCC breaks in wild type and HRR deficient Chinese hamster mutants.

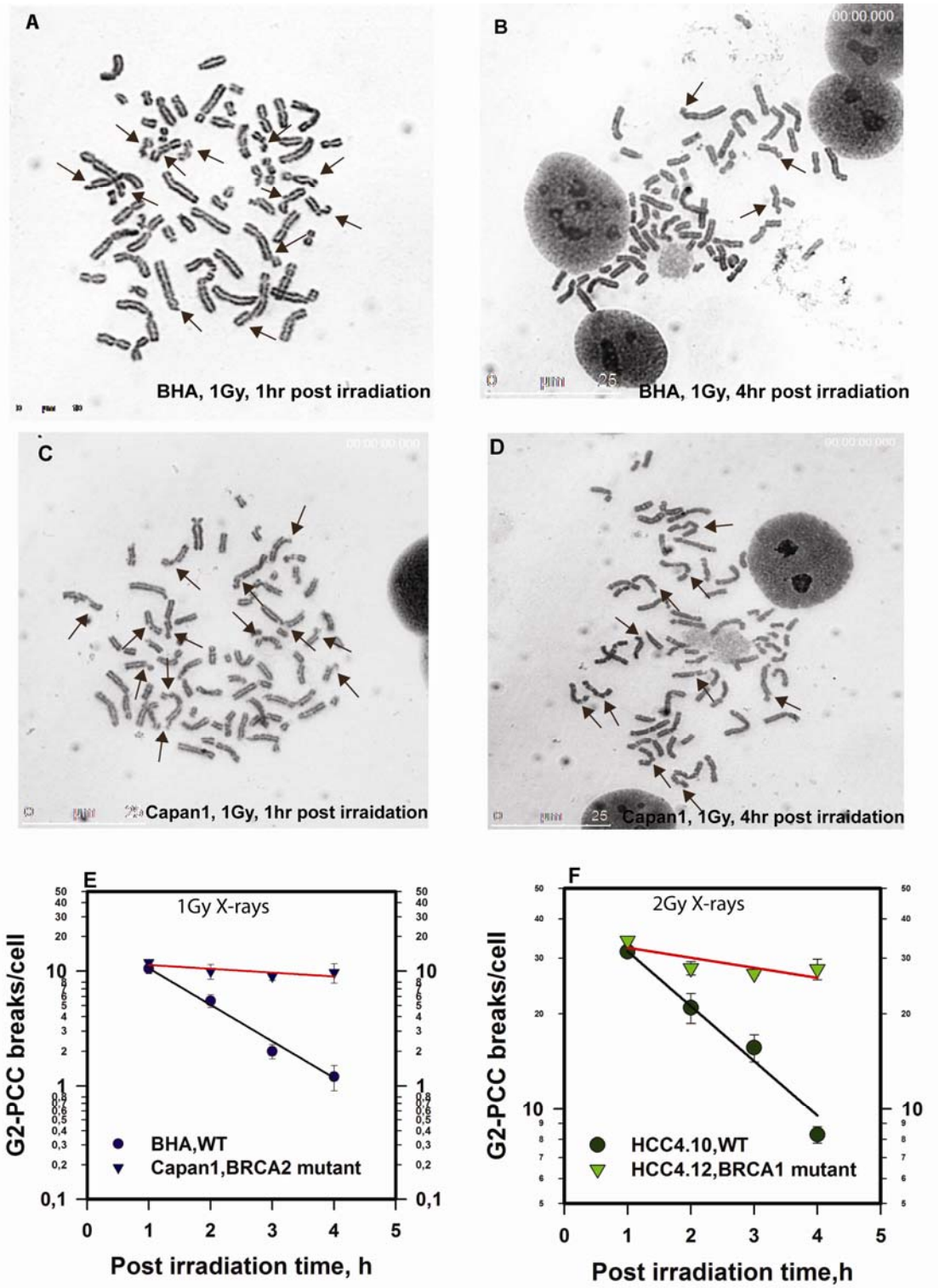


Figure 4.9 Kinetics of calyculin-A induced G2-PCC breaks in exponentially growing wild type, and BRCA1 or BRCA2 deficient human cells. Cells were irradiated with 1 or 2 Gy X-rays and allowed to repair for up-to 4 h. G2-PCCs were induced by adding 50 nM Calyculin-A for 45 minutes and this time is included in the repair time. G2-PCC breaks/cell is plotted against time. (A, B, C, D) Calyculin A induced G2-PCC breaks in BHA and Capan1 cells at different post irradiation times, arrows represent PCC-breaks, (E) Kinetics of G2-PCC breaks in BHA and BRCA2 mutant human pancreatic tumor cells, (F) Kinetics of G2-PCC breaks in wild type and BRCA1 mutant breast cancer cells.

4.4.2 Study of G2-checkpoint activation and recovery in wild type, D-NHEJ and HRR mutants

Checkpoint activation alters the normal distribution of cells through the cell cycle, which can be easily detected by DNA content variations in flow cytometry analysis. This technology is employed here to determine the accumulation of irradiated cells at the G2-M border. Mitotic index (MI) variations also reflect the G2 checkpoint activation and provide a higher sensitivity of detection than G2 phase accumulation. Histone H3 is phosphorylated at Serine-10 (Histone-H3 pS10) early in mitosis and, therefore an antibody against phospho-histone H3 can be used to assess the IR-induced G2 checkpoint delay or entry into mitosis (187). Histone-H3 pS10 staining is very specific for mitotic cells (Figure 4.10 A and Figure 4.11 A, page 59 and page 60 respectively).

4.4.2.1 G2-M accumulation after IR in wild type and repair deficient cells

Exponentially growing wild type, D-NHEJ and HRR deficient hamster cells are irradiated with 2 Gy X-rays and the G2-M fraction is determined by flow-cytometry for up to 24 h post irradiation. During analysis we determined the relative G2-M fraction at each time point, instead of taking the absolute % of G2-M cells. This normalization is done through dividing the %G2-M fraction at any given time by the %G2-M fraction of the corresponding un-irradiated control. Results with CHO cells are summarized in Figure 4.10 B (page 59). CHO cells show strong accumulation in G2-M border, which reaches a maximum (at ~4 times the 0 h value) at 6 h post irradiation, indicating that cells stopped in G2 as a result of the activation of the G2-checkpoint. Subsequently, the percent G2-M fraction starts decreasing and reaches normal levels at 24 h, indicating the recovery from the G2-M checkpoint. D-NHEJ deficient xrs5 cells show ~2.5 times maximum increase at 6 h and recover within 24 h. In contrast, irradiated, HRR deficient irs1SF cells show almost no accumulation in G2-M. The results with wild type V79 and HRR deficient irs1 cells on the other hand show ~ 2 times increase in G2-M fraction at 8 hours, and recovery to normal levels within ~16 h (Figure 4.10 C, page 59). Thus, the G2-checkpoint defect in HRR deficient cells may be cell line/mutation specific.

4.4.2.2 Analysis of the G2-checkpoint in Chinese hamster cells through measurement of mitotic index (MI)

As described in the section 4.4.2.1 (page 56), flow-cytometric determination of % G2-M fraction gives information of G2-checkpoint activation and recovery, but the method is not sensitive enough to allow firm conclusions. As a next step, we studied therefore the G2-checkpoint by determining mitotic index through flow-cytometry. This type of measurement allows evaluation of the kinetics of cell recovery or escape from the G2 checkpoint and reflects for short post-irradiation times exclusively the response of cells irradiated in G2. As a result a direct comparison with the kinetics of chromosome break repair is possible.
In these experiments we used a wide spectrum of Chinese hamster wild type, D-NHEJ and HRR mutants, as well their corrected counterparts. Exponentially growing cells were irradiated with 1 Gy X-rays and the mitotic index was determined for up-to 5 h post irradiation by staining for Histone-H3 pS10. This staining allows the estimation of the % of mitotic cells at each time point.

CHO cells show a rapid decrease in the % mitotic cells within 1 h post irradiation (~90% decrease), which is maintained up-to 2 h. Subsequently the % mitotic cells increases and reaches 100% at 4 h post-irradiation. D-NHEJ deficient xrs5, XR1 and V3 cells show the similar rapid decrease in % mitotic cells and even prolonged delay in recovery, which resembles the stronger G2-checkpoint in these cells (Figure 4.10 D, page 59). When compared with wild type CHO cells, HRR deficient cells irs1SF and Rad51D1 show remarkable defects in G2 checkpoint activation and recovery. The corrected irs1SF cells (Clone X5-1) show almost normal decrease and recovery of % mitotic cells (Figure 4.10E, page 59). See Figure 4.12 (page 61) for a comparison of density plots between CHO, irs1SF and V3 cells.

To further check the generality of these results we employed HRR deficient Chinese hamster fibroblast cells irs1, irs2 and irs3 along with wild type V79 and corrected clone irs1 cells. BRCA2 mutant V-C8 cells are also employed. V79 cells show maximal decrease in % mitotic cells (~80% decrease) at 1 h post irradiation and rapidly resume normal levels within 2 h. irs1 cells show a remarkable defect in G2-checkpoint activation and recovery when compared with wild type V79 and the corrected clone1 (Figure 4.10 F, page 59). irs2, irs3 and V-C8 cells also show clear

defects in G2-checkpoint activation (Figure 4.10 G, page 59). These results suggest that Rad51 dependent HRR is essential for the full development of the G2-checkpoint. They also provide an explanation for the lack of repair of chromatid breaks in HRR deficient cells. This is the first report showing a connection between HRR and G2-checkpoint activation.

4.4.2.3 Study of the G2-checkpoint in BRCA1 and BRCA2 deficient human cells by measuring mitotic index (MI)

We investigated G2-checkpoint response in HRR deficient human cells lines. BRCA2 deficient cells do not show any difference in reduction and recovery of % mitotic cells when compared with their vector control BHA. But interestingly they show significant G2-checkpoint defect when compared with A549 human tumor cells (Figure 4.11 B, page 60). BRCA1 deficient cells show a clear defect in G2-checkpoint activation in comparison to their vector control and A549 cells (Figure 4.11C, page 60). MEFs homozygous for a targeted deletion of exon 11 of the Brca1 gene have been shown to maintain an intact G1-S checkpoint but are defective in the IR-induced G2-M checkpoint (86). Reduced G2-arrest was observed in irradiated cells of breast cancer patients as compared to normal controls (198).

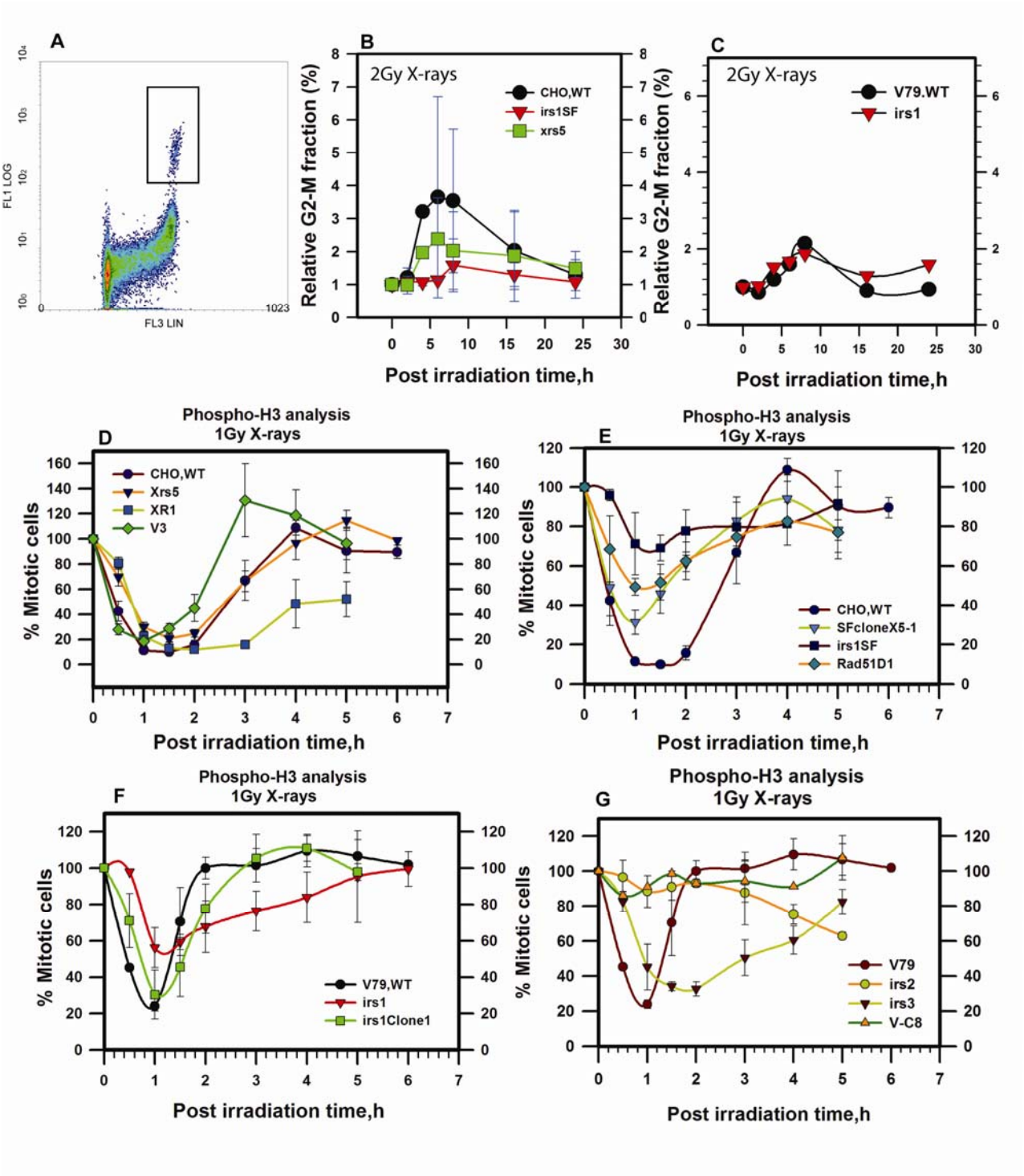


Figure 4.10 Study of G2-checkpoint activation, (A) Density plot displaying Histone-H3 pS10 positive (mitotic) population inside the rectangle, (B and C) G2-M fraction as determined by calculating DNA content using flow-cytometry after exposure to 2 Gy X-rays, (D) Mitotic index determination in D-NHEJ deficient cells, (E) Mitotic index determination in HRR deficient CHO mutants, (F and G) Mitotic index determination in HRR deficient Chinese hamster fibroblast mutants. Exponentially growing cells are irradiated with 1 Gy X-rays for mitotic index determination.

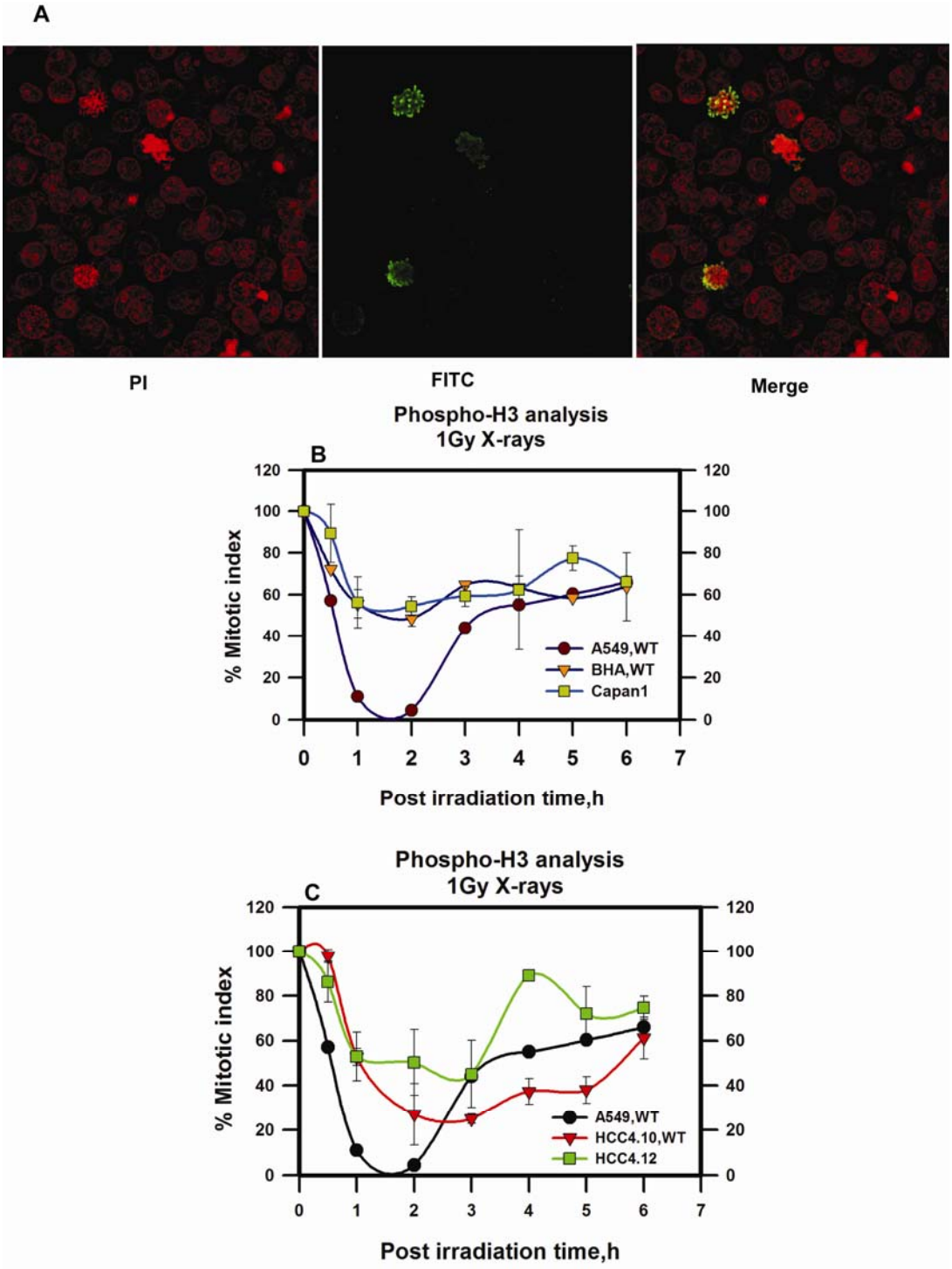


Figure 4.11 Study of G2 checkpoint in wild type, BRCA1 and BRCA2 deficient human cells by flow cytometry. Exponentially growing cells are irradiated with 1 Gy X-rays and mitotic index is determined for up-to 5 h postirradiation. Histone H3-pS10 staining was employed. (A) Confocal image showing Histone H3-pS10+ve mitotic cells [FITC, Green], non mitotic cells are stained with PI only [Red], (B) Mitotic index determination in wild type and BRCA2 mutant cells, (C) Mitotic index determination in wild type and BRCA1 mutant cells.

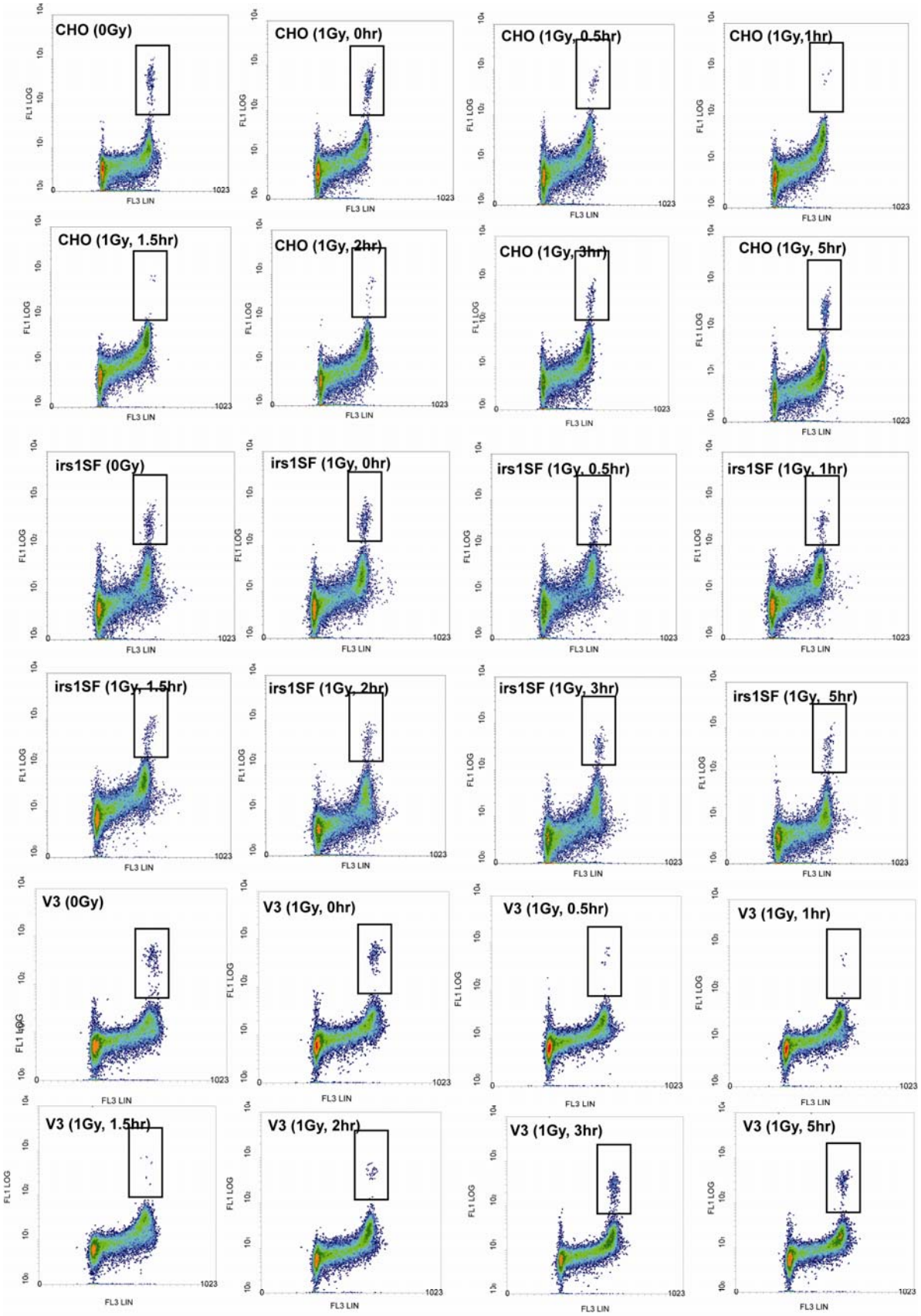


Figure 4.12 Study of G2-checkpoint activation and recovery in wild type, HRR and D-NHEJ mutants of Chinese hamster ovary origin. Exponentially growing cells are irradiated with 1 Gy X-rays and mitotic index is determined by flow cytometry at different post-irradiation times, as indicated in the density plots. Histone-H3 pS10 staining is used as a marker of the mitotic population (indicated inside the rectangle in the density plots).

Table 4.1: Residual chromatid breaks at 5 h post irradiation (% of initial breaks), and half times of repair of chromosome breaks in different Chinese hamster WT, NHEJ and HRR mutants irradiated with 1 Gy X-rays

S/No.	Species	Cell line name	Mutation	Residual chromatid breaks (% of initial breaks)	Half Repair time $T_{1/2}$ (h)
1	Chinese hamster	CHO10B4	WT	10%	2 h
2	Chinese hamster	Xrs5	Ku80	11.8%	2.5 h
3	Chinese hamster	V79	WT	7.5%	2 h
4	Chinese hamster	Irs20	DNA-PKcs	7.6%	2 h
5	Chinese hamster	Irs20k147E	Corrected for DNA-PKcs	10%	2 h
6	Chinese hamster	XR1	XRCC4	14.7%	3.3 h
7	Chinese hamster	Irs1SF	XRCC3	78%	(∞)
8	Chinese hamster V79	Irs1tor	XRCC2	81%	(∞)
9	Chinese hamster V79	Irs1clone1	Corrected for XRCC2	12.7%	2 h

Table 4.2: Residual chromatid breaks at 5 h post irradiation (% of initial breaks) and half times of repair in different MEF WT, NHEJ and HRR mutants irradiated with 1 Gy X-rays

S/No.	Species	Cell line name	Mutation/knockout	Residual chromatid breaks (% of initial breaks)	Half Repair time $T_{1/2}$ (h)
1	Mouse	MEFLig4+/+	WT	16%	2.4 h
2	Mouse	MEFLig4-/-	Lig4	14%	3 h
3	Mouse	MEF347E	Rad54	51.8%	4.5 h
4	Mouse	MEF347B	Lig4 & Rad54	71%	(∞)

Table 4.3: Residual chromatid breaks at 5 h post irradiation (% of initial breaks) and half times of repair in different human WT, NHEJ and HRR mutants irradiated with 1 Gy X-rays

S/No.	Species	Cell line name	Mutation/ knockout	Residual chromatid breaks (% of initial breaks)	Half Repair time $T_{1/2}$ (h)
1	Human	BHA	WT	17.6%	2.7 h
2	Human	Capan1	BRCA2	78.5%	(∞)
3	Human	HCC4.10	WT	21%	3 h
4	Human	HCC4.12	BRCA1	85%	(∞)
5	Human	M059K	WT	2.15%	1.7 h
6	Human	M059J	DNA-PKcs	22%	3.5 h
7	Human	HCT116	WT	17.6%	3 h
8	Human	HCT116	DNA-PKcs	31%	3.2 h
9	Human	HCT116	Lig4	30%	4 h

Table 4.4: Residual chromatid breaks at 5 h post irradiation (% of initial breaks) and half times of repair in different cells deficient in important DDR signalling proteins irradiated with 1 Gy X-rays

S/No.	Species	Cell line name	Mutation/ knockout	Residual chromatid breaks (% of initial breaks)	Half Repair time $T_{1/2}$ (h)
1	Human	M059K	WT	2.15%	1.7 h
2	Human	M059J	DNA-PKcs	22%	3.5 h
3	Mouse	H2AX+/+	WT	16%	2.5 h
4	Mouse	H2AX-/-	H2AX-/-	18.3%	3 h
5	Mouse	53BP1+/+	WT	16%	2.5 h
6	Mouse	53BP1-/-	53BP1-/-	20%	2.5 h
7	Human	M059K	WT	2.15%	1.7 h
8	Human	AT5BIVA	ATM	45%	4.2 h
9	Human	NBS1 LB	Nbs1	62%	(∞)
10	Human	GM-847	WT	14.5%	1.5 h
11	Human	GM-847ATRkd	ATR kinase dead	93%	(∞)

Table 4.5: Residual chromatid breaks at 5 h post irradiation (% of initial breaks) and half times of repair in DNA-PKcs deficient human cells treated with different inhibitors before irradiation with 1 Gy X-rays

S/No.	Species	Cell line name	Mutation/ knockout	Treatment	Residual chromatid breaks (% of initial breaks)	Half Repair time $T_{1/2}$ (h)
1	Human	M059K	WT	1Gy	2.15%	1.7 h
2	Human	M059J	DNA-PKcs	1Gy	22%	3.5 h
3	Human	HCT116	WT	1Gy	17.6%	3 h
4	Human	HCT116	DNA-PKcs	1Gy	31%	3.2 h
5	Human	HCT116	DNA-PKcs	10uM Ku55933+1Gy	67%	(∞)
6	Human	HCT116	DNA-PKcs	4mM caffeine +1Gy	46.12%	(∞)
7	Human	M059J	DNA-PKcs	10uM Ku55933+1Gy	77.9%	(∞)
8	Human	M059J	DNA-PKcs	Transitional CtIP knock down+1Gy	97.6%	(∞)
9	Human	M059J	DNA-PKcs	4mM caffeine+1Gy	75.7%	(∞)
10	Human	M059J	DNA-PKcs	10uM PJ34+4mM caffeine+1Gy	81%	(∞)
11	Human	M059K	WT	4mM caffeine+1Gy	16.6%	2 h
12	Human	M059K	WT	10uM PJ34+4mM caffeine+1Gy	11.4%	2.2 h

5. Discussion

5.1 The role of D-NHEJ in the repair of G2-chromosomal breaks

D-NHEJ is an efficient but error-prone repair process that directly rejoins broken DNA ends. Although it restores integrity in the DNA, it is not equipped to restore the sequence in the vicinity of the DSB (199) and results frequently in the loss of genetic information. Furthermore, as the end joining process is indiscriminate and there are no mechanisms in place to ensure the rejoining of the original ends, chromosomal rearrangements ensue when the ends of unrelated molecules/chromosomes are joined together (200).

For the human and the mouse immune systems, D-NHEJ is the central DNA repair mechanism supporting V(D)J recombination (118). Despite its error-prone nature, D-NHEJ is considered the main pathway of DSB repair in higher eukaryotes. One of the main reasons for this development may be that mammalian genomes are large and the first preference after breakage is to restore integrity using a fast repair process such as D-NHEJ.

Our results of the repair kinetics of chromatid breaks in D-NHEJ mutants support this notion, as in the absence of functional D-NHEJ; more DSBs are converted to chromatid breaks (~2 to 3 fold increase as compared to wild type cells). This shows that D-NHEJ is important in maintaining genomic integrity in mammalian cells via repair of the bulk of DSBs. On the other hand, no defect is observed in the kinetics of chromatid breaks in D-NHEJ deficient cells, as in the metaphases scored for up to 5 h after irradiation, their numbers decrease with kinetics similar to those measured in wild type cells. This observation suggests that D-NHEJ is not contributing to the repair of the subset of DSBs causing G2 chromosome breaks. In addition, since the mitotic index drops significantly after irradiation in D-NHEJ mutants, the unchanged repair kinetics also reflect only a subset of the cells that would have entered mitosis in the absence of radiation. More work and further analysis will be required to clarify these issues and address the real contribution of D-NHEJ to chromosome break repair. The results with D-NHEJ deficient cells contrast those of HRR deficient cells, where a strong reduction in the ability to repair G2 chromosome breaks was observed.

5.2 HRR is required for the repair of G2-chromosomal breaks

HRR is an error free pathway for the repair of DSBs (201), which prevents chromosomal deletions and rearrangements. RAD51 paralogs act as genomic caretakers by playing a central role in HRR. They are involved in DNA damage recognition, strand invasion into an undamaged DNA template, branch migration and resolution of Holliday junctions (202,203). Rad51 paralogs are clearly implicated in the prevention of *HPRT* gene mutations and *DHFR* gene amplification, which lead to carcinogenesis (204). Many proteins involved in HRR are products of hereditary cancer predisposition genes, implying that failure to adequately regulate HRR, and the consequent genomic instability, plays a causal role in cancer.
The critical role of HRR in suppressing genomic instability is reflected in the early embryonic lethality of mice lacking Rad51, BRCA1 or BRCA2. The abundance of chromatid type of aberrations observed in such mutant cells suggests that the major function of these genes is to control sister chromatid recombination (205,206). The reduced Rad51 foci formation and severely impaired HRR in Rad51 paralog mutants also suggests an important role for the paralogs in assisting Rad51 during HRR (207).

Mammalian cells deficient in HRR paralogs have been shown to be significantly radiosensitive (167,168). The relatively high radio-resistance of NHEJ-defective mutants in the late S/G2 portion of the cell cycle further suggests that HRR promotes survival when sister chromatids are present (169,170). While the importance of HRR has been clearly shown for the repair of site directed chromosomal double strand breaks induced by the endonuclease *I-SceI* (177,178), its contribution to the repair of IR induced DSBs remains controversial (173-175). HRR does not seem to play any detectable role in the repair of the bulk of DSBs induced by IR even in the G2-phase of the cell cycle (122).

Despite the fact that HRR is important for genomic stability, its role in the repair of IR induced DSB is unclear. Our results of the kinetics of chromatid breaks in HRR deficient mammalian cells clearly demonstrate the absolute necessity of HRR for the repair of G2-chromosomal breaks in a species independent manner. This means that HRR is responsible at a minimum for the repair of those few specific DSB, which are the precursors of chromosome breaks. Due to their low numbers, these DSBs may

remain undetected by physical methods of measuring DSB repair like Pulsed field gel electrophoresis (PFGE).

Although D-NHEJ is shown to be functional throughout the cell cycle, it does not seem to take part an obvious to the repair of G2 chromosome breaks, even when HRR is not functional. This suggests that that these DSBs are specifically processed by the error free HRR pathway. This may be highly appropriate when considering that un-repaired or miss-repaired chromosomal breaks may lead to cell death. Also sequence restoration at these damaged sites may also be an additional essential requirement.

These are the first results showing a direct role of HRR in the repair of IR induced chromosomal breaks. One possible mechanism underlying this pathway selection is that DNA ends are resected in G2 phase to produce 3' single stranded tails, which initiates HRR. The same processes may exclude the NHEJ repair machinery from occupying the DNA ends (208,209). When HRR is initiated but not completed due to defects in key proteins like Rad51 paralogs, BRCA1 and BRCA2 (202,203); a complete inhibition of chromosome repair is observed. Thus the un-repaired chromosome breaks seen in our experiments most likely to reflect DSBs whose repair by HRR was abrogated. The properties of this fraction of DSBs remain to be elucidated.

5.3 The impact of DDR signalling on HRR mediated repair of G2-chromosomal breaks

5.3.1 ATR and NBS1 are more important than ATM in HRR mediated repair of G2-chromosomal breaks

In the canonical model of DSB signalling ATM is considered as the primary kinase responsible for detecting and signalling this form of DNA damage (210), while ATR is thought to signal for UV induced damage and stalled DNA replication forks (61,63,211). Although ATM has long been implicated in signalling IR-induced DSBs, new data suggest that ATR also functions in IR induced DNA damage signalling (60,62,80,212). A novel concept of ATM dependent ATR activity has also been advanced by various groups (213-217).

Mutation in BRCT, FHA or MRE-11 binding domain of *NBS1* decreased HRR activity as measured by a GFP based HR assay. NBS cells expressing these mutated NBS1 forms cannot form DSB-induced MRE11 foci. These results indicate that the recruitment of MRE11 to the DSB sites by NBS1 is important for HRR activity. On the other hand, mutation in ATM-phosphorylating of ATM-binding sites did not influence HRR activity. Moreover, AT cells showed HRR activity at a similar level as ATM complemented cells, suggesting that ATM might be dispensable for HRR (110).

Our results on the kinetics of chromatid breaks in the *ATM* mutants, AT5BIVA and cells expressing kinase dead ATR show that while repair of chromatid breaks is significantly compromised in *ATM* mutants (~ 45% residual damage), it is inhibited almost completely in ATRkd cells with ~ 93% residual damage (See section 4.3.2, page 46). One should consider that ATRkd cells are unable to repair chromatid breaks in spite of having normal ATM levels, while AT5BIVA cells could repair ~ 55% breaks, with normal ATR levels. These results suggest that ATR plays a more important and indispensable role than ATM in HRR. This is in line with a previous report where ATR/Chk1 over-expression was shown to prolong G2 accumulation and to increase survival of ATM deficient cells (85). Recent work carried out in our laboratory also shows an indispensable role of ATR in the activation and maintenance of IR induced G2-checkpoint (Fan, X unpublished data). Nbs1 mutant cells also show a stronger phenotype than ATM in terms of HRR mediated repair of G2-chromosomal breaks (See section 4.3.3, page 47).

5.3.2 H2AX and 53BP1 deficiency does not affect HRR mediated repair of G2-chromosomal breaks

H2AX and 53BP1 are clearly implicated in the early response to IR induced DSBs. H2AX has been shown to contribute to HRR in an MDC1 dependent manner, while 53BP1 has been shown to be involved in XRCC4 dependent NHEJ (107,217).
Our results of chromatid break kinetics in H2AX and 53BP1 knockout cells show normal repair of chromatid breaks. Results with 53BP1 knockout cells confirm its role in pathways other than HRR but results with H2AX knockout cells are surprising as no defect can be observed on HRR mediated repair of G2-chromosomal breaks (See

section 4.3.1, page 46). However involvement of this protein in HRR has been reported for the repair of *I-SceI* induced DSBs (107).

5.4 Influence of G2-checkpoint on HRR mediated repair of G2-chromosomal break

5.4.1 The G2-checkpoint plays an important role in the repair of G2 chromosomal breaks

Our G2-PCC results in Chinese hamster and human cells deficient in HRR clearly show the influence of the G2-checkpoint on the repair of chromatid breaks. While a similar level of initial damage is induced in wild type and HRR mutants, the majority of PCC breaks remain un-rejoined in the latter (See section 4.4.1, page 53). These results suggest that the G2 checkpoint facilitates the repair of chromosome breaks in wild type cells but it does not seem to work properly in the absence of HRR.

5.4.2 Proper G2-checkpoint response is dependent on functional HRR

Classically, DSB repair and cell cycle checkpoints are considered as two different means for maintaining genomic stability (218-221). In higher eukaryotes, the signalling response to DSBs is centred on ATM, ATR and DNA-PK. These PI3K kinases trigger cell cycle arrest following DNA damage, and are thought to provide in this way time for the cells to repair. While ATM and ATR act as initiators of DNA damage checkpoint signalling (222), DNA-PKcs has a critical role in DSB repair via NHEJ (200). The breast cancer associated gene BRCA1 has also been shown to be required for G2 checkpoint control, as well as for HRR mediated repair of DSBs (86,87). ATM defects impair HRR-mediated DSB repair and may link cell cycle checkpoints to HRR activation (223).

Our results on the G2 checkpoint using Histone-H3 pS10 staining reveal a novel role of the HRR machinery in the activation and maintenance of this checkpoint. Chinese hamster cells deficient in *Rad51* paralogs *Rad51B*, *Rad51C*, *Rad51D*, *XRCC2* and *XRCC3* as well in BRCA2 show a remarkable defect in G2-checkpoint activation, while *Ku80*, *DNA-PKcs* and *XRCC4* deficient cells show strong G2-checkpoint activation and delayed recovery as compared to WT cells (See section 4.4.2.2, page 57). The XRCC2 and XRCC3 complemented cells resume G2- checkpoint activation

and recovery to levels similar to WT cells. These results link HRR to the IR induced G2-checkpoint signalling and impose a requirement for HRR function to achieve full G2-checkpoint activation. This requirement goes over and above the requirement for DDR signalling proteins ATM and ATR, which have long been shown to be associated with G2-checkpoint responses. The strong G2-checkpoint activation and delayed recovery in NHEJ deficient cells suggests that this delay in cell cycle progression facilitates repair via HRR in these cells.

These results provide the possible explanation for the lack of G2-chromosomal break repair in HRR deficient cells and suggest that checkpoints and repair pathways do not work independently, but co-operate instead to ensure genomic stability.

How signalling proteins like ATM and ATR fail to activate G2-checkpoint in HRR deficient cells, needs to be investigated in detail. Our results of chromatid break kinetics in ATM mutant and ATRkd cells, as well as recent data from another study from our lab (Fan, X unpublished data) implicate ATR in this response.

6. Conclusions

Our results show absolute requirement for HRR in the repair of G2 chromosomal breaks and emphasize its importance in the activation and maintenance of the G2-checkpoint.

While D-NHEJ is responsible for the repair of majority of IR induced DSBs in mammalian cells, HRR is critically involved in the repair of a fraction of DSBs which can be converted to chromatid breaks. If left unrepaired or if misrepaired, these DSBs can compromise cell survival and initiate genomic instability.

Defects in DDR signalling proteins that are also implicated directly or indirectly in HRR show a strong inhibition in the repair of G2-chromosomal breaks. Notably, ATR plays an indispensable role in the repair of these breaks.

Full G2-checkpoint activation after IR exposure is dependent on functional HRR machinery. These results suggest that the purpose of G2-checkpoint activation is more likely to facilitate repair of the fraction of DSBs that are processed by HRR and thus require more time for their repair, as HRR is considered to be a slow process when compared to D-NHEJ.

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8. Appendix

Appendix 1: Buffers and solutions

1. 1X Phosphate Buffered Saline (PBS)

Dissolve the following in 800ml Double distilled water (ddH2O).

• 8 g of NaCl

• 0.2 g of KCl

• 1.44 g of Na2HPO4

• 0.24 g of KH2PO4

Adjust pH to 7.4.

Adjust volume to 1 L with additional ddH2O.

Sterilize by autoclaving and store at 4°C.

2. Trypsin-EDTA (Trypsin0.05%, EDTA 0.02%)

0.5 g of Trypsin

0.2 g of EDTA

Adjust the volume to 1 L with 1X PBS.

Sterilize by passing through 0.22 µm filter and store at -20°C

3. 100X Propidium iodide. (4 mg/ml)

400mg of PI

Adjust the volume to 100 ml with ddH2O.

Store at -20°C in dark.

4. 100X RNase (6.2 mg/ml)

620 mg of RNase

Adjust the volume to 100 ml with ddH2O

Store at -20°C in dark.

5. FACS Permeabilization solution (PBS + 0.25% Triton X-100)

2.5 ml Triton X-100

Adjust the volume to 1000 ml with PBS.

Store the solution at RT in dark.

6. FACS blocking buffer (PBS + 0.05% Tween-20 + 1% BSA)

10 g BSA

2.5 ml 20% Tween-20

Adjust the volume to 1000 ml with PBS.

Store at -20°C.

7. Hypotonic solution

0.56 g KCl

Adjust the volume to 100ml with ddH2O

Prepare fresh at the time of experiment

8. Carnoy's fixative (3 parts Methanol + 1 part acetic acid)

75 ml methanol

25 ml acetic acid

Prepare fresh at the time of experiment

Appendix 2: Settings for cell cycle analysis

Cell cycle analysis programme settings

Flow cytometry: COULTER EPICS XL

Sheath Speed: middle

Total cells sampled: 20000

Maximal running duration: 900 seconds

Working mode: carousel

Excitation (nm): 488

Filter spectrum (nm): 655-735

FS-PMT (volt): 55

FS-Gain: 2

SS-PMT (volt): 400

SS-Gain: 1

AUX-PMT (volt): 300

AUX-Gain: 2

PI-PMT (volt): 530

PI-Gain: 2

Discriminator: PI>3

Gate: single cell

Appendix 3: Settings for bivariate flowcytometry

Flow cytometry: COULTER EPICS XL

Sheath Speed: middle

Total cells sampled: 20000

Maximal running duration: 900 seconds

Working mode: Manual

Program: DNA-PI-H3ps10-FITC

FS-PMT (volt): 55

FS-Gain: 2

SS-PMT (volt): 400

SS-Gain: 1

AUX-PMT (volt): 300

AUX-Gain: 2

Excitation of PI: 488nm

PI- Filter spectrum: 655-735nm

PI-PMT (volt): 520

PI-Gain: 2

PI signal: Linear

PI-GATE: single cell

Discriminator: PI>3

Excitation of FITC: 488nm

FITC Filter: 490-550nm

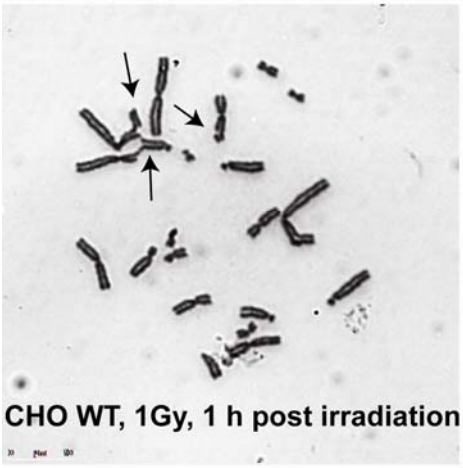
FITC-PMT (volt): 520

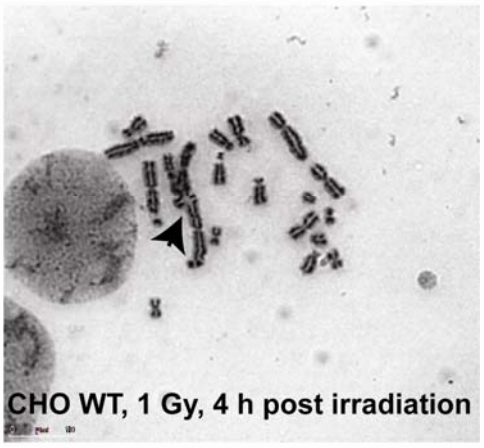
FITC-Gain: 2

FITC signal: Logarithmic

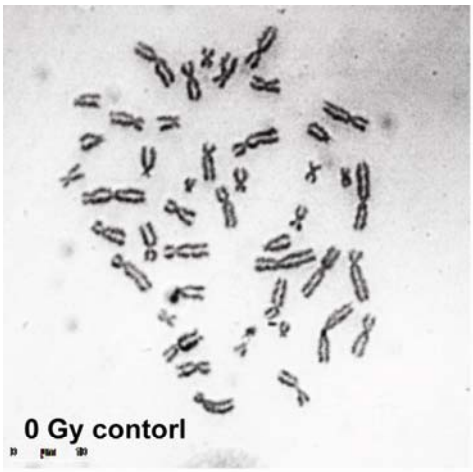
FITC-GATE: FITC positive

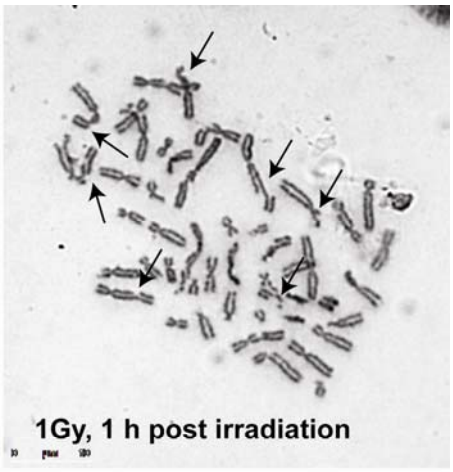
Appendix 4: Chromatid breaks and exchanges in CHO cells irradiated with 1Gy X-rays (arrows represent chromatid breaks and arrow heads represent chromatid exchanges)

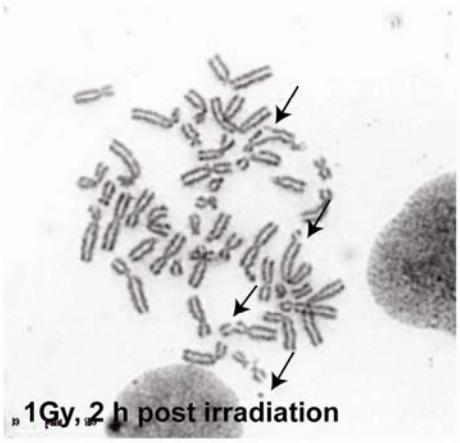


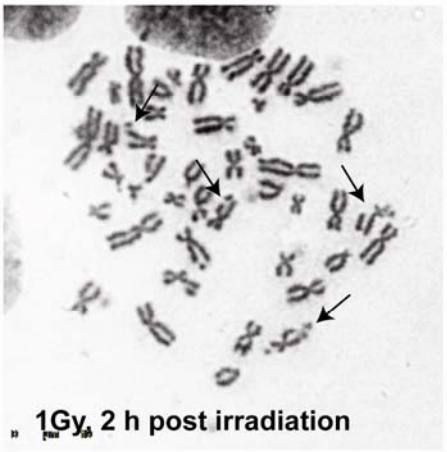


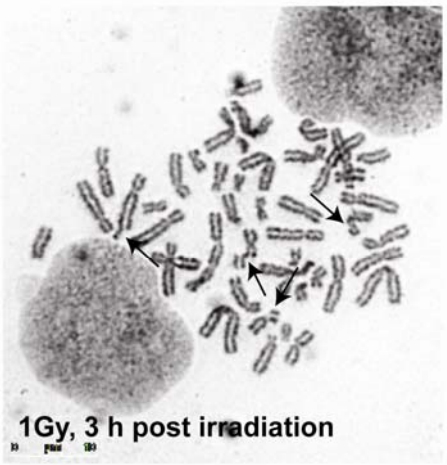
Appendix 5: Chromatid breaks in human cells irradiated with 1Gy X-rays (arrows represent chromatid breaks)

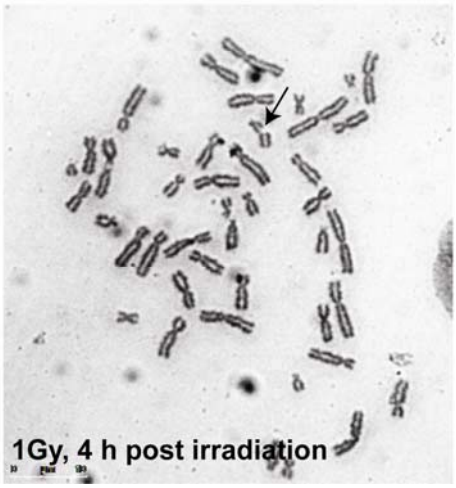












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10. Curriculum Vitae

Aashish Soni

Institute for Medical Radiation Biology
Universitätsklinikum Essen
Hufelandstr. 55
Essen 45147, Germany

Phone: + 49-201-723-4156 (Office)
+ 49-17620701522 (Mobile)
E-mail: aashish.soni@uk-essen.de

PERSONAL DETAILS:

Nationality : Indian

Date of birth : 16th September 1979

Sex : Male

Academic Qualifications:

1. **PhD project (since March, 2007)**

PhD Program : DFG Graduate Training Program 1431/1 "Transcription, Chromatin structure and DNA repair in development and differentiation

Organization : Institute for Medical Radiation Biology, University Duisburg-Essen medical School

Supervisor : Prof. Dr George Iliakis

2. **Junior Research fellow (JRF) (February 2, 2005 to October 31, 2006)**

"Preclinical investigation on cancer therapy potential of Ashwagandha (*Withania somnifera*)"

Organization **:** Dept. of Research, Jawaharlal Nehru Cancer Hospital and Research Centre, Bhopal, Madhya Pradesh, India.

Supported by Department of Science and Technology, Government of India (Project No.SP/SO/B61/98)

Supervisor **:** Prof. Dr. P. Uma Devi

3. PG Diploma in Genetic engineering and Bioprocess development (2004 – 2005)

Organization **:** Genome Research Centre, Department of Microbiology and Biotechnology Centre, MS University, Baroda, Gujarat, India.

Note: Joined the course as a regular student on August 1, 2004 and got the training up to February 1, 2005, but could not able to complete the course because of the selection as Junior Research Fellow in a DST project sanctioned to Dr. P. Uma Devi at Jawaharlal Nehru Cancer Hospital and Research Centre, Idgah hills, Bhopal, India.

4. Master of Science (M.Sc.) in Applied Microbiology & Biotechnology (2001-03)

Organization **:** Dr. Harisingh Gour University, Sagar, Madhya Pradesh, India

5. **Bachelor of Science (B.Sc.) in Industrial Microbiology (1999-2001)**

Organization : Dr. Harisingh Gour University, Sagar, Madhya Pradesh, India

Additional Qualifications:

Teaching Experience: Teaching experience as a guest faculty in the Department of Applied Microbiology and Biotechnology, for undergraduate and postgraduate classes at Dr. Harisingh Gour University, Sagar (M.P.) for the academic session 2003-2004.

Conference/Paper/Poster presented:

1. Shraddha Rusia, Aashish Soni and P.Uma Devi, **Ashwagandha Enhances Tumor Growth Inhibition by Cis-Chlorodiamine Platinum (II).** Fifth International Conference on Ethnopharmacology and Alternative medicine & Annual Conference of National Society of Ethnopharmacology. Organized jointly by Amala Cancer Research Centre & Amala Ayurveda Hospital and Research Centre, Thrissur – 680 555, Kerala, India, January 2006.
2. Aashish Soni, Shraddha Rusia and P.Uma Devi, **Withaferin A enhances radiation-induced apoptosis by DNA repair inhibition**. International Conference on Radiation Biology held at department of Medical sciences, Banaras Hindu University, India, November 20th to 22nd 2006.
3. Attended the International Symposium on Chromosomal Aberrations (ISCA, 2009), held at St. Gour Germany, July 11-12, 2009.

11. Publications and conferences

Poster/paper presented

1. Shraddha Rusia, **Aashish Soni** and P. Uma Devi, **Ashwagandha Enhances Tumor Growth Inhibition by Cis-Chlorodiamine Platinum (II).** Fifth International Conference on Ethnopharmacology and Alternative medicine & Annual Conference of National Society of Ethnopharmacology. Organized jointly by Amala Cancer Research Centre & Amala Ayurveda Hospital and Research Centre, Thrissur – 680 555, Kerala, India, January 2006.

2. **Aashish Soni,** Shraddha Rusia and P. Uma Devi, **Withaferin A enhances radiation-induced apoptosis by DNA repair inhibition**. International Conference on Radiation Biology held at department of Medical sciences, Banaras Hindu University, India, November 20th to 22nd 2006. The abstract was published in a special issue of Indian Journal of Radiation Research, volume 3, November 4, 2006 (ISSN 0973-0168).

12. Declaration

Erklärung:

Hiermit erkläre ich, gem. § 6 Abs. 2, Nr. 7 der Promotionsordnung der Math.-Nat. Fakultäten zur Erlangung des Dr*. rer. nat*., dass ich das Arbeitsgebiet, dem das Thema: **"*The role of homologous recombination repair in the processing of G2-chromosomal breaks and maintenance of G2-checkpoint*"** zuzuordnen ist, in Forschung und Lehre vertrete und den Antrag von **Herr Aashish Soni** befürworte.

Essen, den …….

Unterschrift d. Wissenschaftl. Betreuers/Mitglieds
der Universität Duisburg-Essen

Erklärung:

Hiermit erkläre ich gem. § 6, Abs. 2, Nr. 6 der Promotionsordnung der Math.-Nat. Fakultäten zur Erlangung des Dr*. rer. nat*., dass ich die vorliegende Dissertation selbständig verfasst und mich keiner anderen als der angegebenen Hilfsmittel bedient habe.

Essen, den …….

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Erklärung:

Hiermit erkläre ich gem. § 6, Abs. 2, Nr. 8 der Promotionsordnung der Math.-Nat. Fakultäten zur Erlangung des Dr. *rer. nat*., dass ich keine anderen Promotionen bzw. Promotionsversuche in der Vergangenheit durchgeführt habe und dass diese Arbeit von keiner anderen Fakultät abgelehnt wurden ist.

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